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GRANT NUMBER DAMD17-94-J-4427

TITLE: Effect of Estrogen on Progression of Human Proliferative Breast Cancer Disease in a Xenograft Model

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REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (1 Aug 97 - 31 Jul 98)

4. TITLE AND SUBTITLE
Effect of Estrogen on Progression of Human Proliferative Breast Disease in a Xenograft Model

5. FUNDING NUMBERS
DAMD17-94-J-4427

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Michigan Cancer Foundation
Detroit, Michigan 48201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Utilizing the T24-*Ha-ras* transfected MCF10A xenograft model of early human breast cancer progression we have 1) demonstrated the direct effects of estrogen on growth and sequence of progression of human preneoplastic breast disease, 2) demonstrated the utility and validity of bcl-2, cyclin D1, and c-erbB2 as specific markers for breast cancer progression, 3) established an *in vitro* assay system that recapitulates *in vivo* growth and organization of MCF10AT cells into highly proliferative estrogen responsive ductular elements. Experments analyzing chemotherapeutic and chemopreventive effects of tamoxifen on growth and progression of MCF10AT lesions are underway. Our data thus far show that transcriptional activation of estrogen receptor gene is not a result of alteration in methylation status of estrogen receptor gene or a position effect of insertion of exogenous T24 *Ha-ras* gene.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	49
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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P.V. Allaire Sullivan — PI - Signature

PVS 8/27/98 — Date

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PROGRESS REPORT OF WORK ACCOMPLISHED BY P.I. IN YEAR FOUR AT KARMANOS CANCER INSTITUTE

This report is not a final report since the grant has been awarded extension for an additional year ending August 31, 1999.

BACKGROUND

The MCF10AT system is a xenograft model in which the progression of a T24 *Ha-ras* transformed derivative of MCF10A, viz., MCF10AneoT, can be followed from a histologically precancerous stage to development of invasive carcinoma. In contrast to parental MCF10A cells, MCF10AneoT cells form persistent lesions in immunedeficient mice. MCF10AneoT and lines derived by alternating *in vivo* transplantation and *in vivo* culture (MCF10ATn) are collectively known as the MCF10AT system (1). MCF10AT cells grow in nude mice, where over a period of several months, they undergo a sequence of progressive histological changes mimicking those seen in breasts of women at high risk of breast cancer (i.e., atypical hyperplasia) that culminate in a significant proportion of grafts with frankly invasive carcinoma (1). The lesions formed by lines of the MCF10AT system are composed of a heterogeneous spectrum of ductular tissues with a range of morphology that includes mild hyperplasia (grade 1), moderate hyperplasia (grade 2), atypical hyperplasia (grade 3), carcinoma *in situ* (grade 4) and undifferentiated carcinoma (grade 5; Ref. 2). Thus, the MCF10AT system provides a transplantable, xenograft model of human proliferative breast disease with proven neoplastic potential. It is important to note that it is the only human model that has been shown to exhibit the histological stigmata associated in women with high risk of developing breast cancer and, furthermore, to undergo preneoplastic and neoplastic progression *in vivo*. Strategies for breast cancer prevention require understanding of the molecular events leading to transformation or progression of human breast epithelial cells (HBEC). We have utilized the MCF10AT xenograft model of early human breast cancer progression to determine a) the effect of estrogen on growth and on morphologic sequence of progression of benign and premalignant ducts, and b) genetic and cellular changes that accompany (or characterize) progression.

Work accomplished at Karmanos Cancer Institute thus far:

1. We have shown that while MCF10A cells are estrogen receptor (ER) negative, MCF10AT cells are ER positive, i.e., the endogenous ER gene is transcriptionally activated. The activated ER protein in MCF10AT cells is functionally active based on its ability to mediate i) E_2-regulated increase of transcription from both exogenous (ERE-TKCAT), and endogenous E_2-regulated genes, progesterone receptor and pS2, ii) confer estrogen-mediated effects on growth and proliferation. Although our studies thus far have not provided any clues on the mechanisms responsible for transcriptional activation of ER gene, our data do show that it is not a result of 1) alterations in methylation status of ER gene as reported in other ER positive (MCF-7) and ER negative (MDA-MB 435) cells, or 2) of positional insertion of exogenous *T24-Ha ras* gene in MCF10AT cells.

A manuscript reporting these data has been accepted for publication in Int. J of Oncol.
Transcriptional activation of functional endogenous estrogen receptor gene expression in MCF10AT cells: A model for early breast cancer.
P.V.M. Shekhar, M.L.-Chen, J. Werdell, G.H. Heppner, F.R. Miller and J.K. Christman. In press.

2. Results from our studies established the presence of a novel mechanism for functional inactivation of wild type p53 in the absence of genetic alterations. Our data showed not only a correlation between accumulation of conformationally altered wild type p53 and neoplastic behavior of MCF10AT cells, but also that conformationally altered wild type P53 (rather than genetic alterations) is defective in P53-mediated functions such as transcription activation.

Altered p53 conformation: a novel mechanism of functional inactivation of wild type p53 in a model for erly human breast cancer.
P.V.M. Shekhar, R. Welte, J.K. Christman, H. Wang and J. Werdell. Int. J. Oncol., 11: 1087-1094, 1997.

3. Stabilized existence of P53 in a conformationally altered "mutant" immunological phenotype in MCF10AT xenografts appear to result from its interaction with several high molecular weight proteins present in the milieu of MCF10AT cells. Differences in P53 function between parental MCF10A and MCF10AT xenografts appear to correlate with: 1) absence of phosphorylated native p53 in MCF10AT xenografts, 2) presence of high levels of conformationally altered P53 in MCF10AT xenografts, 3) specific interaction of conformationally altered P53 with high molecular weight proteins in MCF10AT xenografts, 4) interaction of native wild type P53 with MDM-2 possibly resulting in functional inactivation in MCF10AT xenografts. Our results also show that while MDM-2 complexes with a subset of wild type P53 (native form), it does not form complexes with conformationally altered P53. Thus, at least two mechanisms may account for loss of P53 function in MCF10AT xenografts: a) predominant existence of P53 in a conformationally altered state, and b) binding of native wild type P53 with MDM-2. We are currently collecting human breast specimens with known grades and stages of progression to conduct similar analysis of p53 structure, phosphorylation status and interaction with MDM-2. Results from these studies will establish the existence of similar mechanisms of functional inactivation of wild type p53 as in MCF10AT cells.

4. We have demonstrated that the observed epidemiologic link between estrogen and increased risk of breast cancer indeed reflects a direct effect of estradiol on growth and on sequence of progression of benign or premalignant ducts. Our results suggest that estrogen exerts a growth promoting effect on benign or premalignant ducts by enhancing a) the frequency of lesion formation, b) size of lesions, c) the speed of transformation from grades 0/1 (simple/mild hyperplasia) to grades 3 (atypical hyperplasia) and higher (carcinoma *in situ* and invasive carcinoma), and d) the degree of dysplasia.

Direct effect of estrogen on sequence of progression of human preneoplastic breast disease.

P.V.M. Shekhar, P. Nangia-Makker, S.R. Wolman, L. Tait, G.H. Heppner and D.W. Visscher. Amer. J Pathol, 152: 1129-1132, 1998.

5. We have also demonstrated the estrogenic properties of organochlorine pesticides and its effects on growth and ER function in preneoplastic MCF10AT cells.

Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines.
P.V.M. Shekhar, J. Werdell and V.S. Basrur. J. Natl Cancer Inst., 89: 174-1782, 1997.

WORK ACCOMPLISHED IN YEAR 4

BODY OF REPORT

Immunohistochemical staining and correlation of specific breast cancer genes with lesion dysplasia: In year 3 of our study, we had reported preliminary data obtained for staining patterns of bcl-2, cyclin D1, c-erbB-2 and pS2 in paraffin embedded sections of MCF10AT1 lesions that were recovered from estrogen-exposed mice. These markers were selected since their expression levels have been correlated to ER status of human breast cancer cell lines. Bcl-2 protein levels are downregulated in MCF-7 cells that are starved of estrogen, resulting in increased bax/bcl-2 ratio and induction of apoptosis. However, restimulation of estrogen-dependent MCF-7 cells with estrogen results in elevated expression of bcl-2 with a concomitant decrease in bax expression (3,4). Cyclin D1 has been recently shown to form functional complexes with ER in the absence of estrogen (5). This interaction results in transcriptional activation of ER-regulated genes. Cyclin D1/ER interaction is inhibited by antiestrogens, and this interaction is independent of cdk4/cdk6 (5). Expression of high levels of c-erbB-2 has been correlated with poor prognosis (6). Expression of pS2 was examined as it is an estrogen-regulated gene, the expression of which requires a functional ER (7).

We have completed analysis of localization and distribution of these specific molecular markers in estrogen-exposed MCF10AT1 lesions. Our findings confirm our preliminary data with regard to the specificity and selectivity of association of bcl-2, erbB-2, cyclin D1 and pS2 distribution with histological grades.

Bcl-2 staining was observed primarily in simple glandular regions and focally in nonatypical hyperplasia, whereas c-erbB-2 protein is absent in simple glands but its expression is increased in higher grades of dysplasia, viz., hyperplasia with atypia, carcinoma *in situ* and invasive carcinoma. Cyclin D1 protein expression is negative in all grades except in regions of lesions showing invasive carcinoma where intense nuclear staining of cyclin D1 was observed. pS2 staining was primarily observed in simple and hyperplastic ducts. Statistical analysis of these data by chi squared test indicated significant correlation ($p<0.05$) of expression patterns for bcl-2, erbB-2 and cyclin D1 with degree of dysplasia. Since MCF10AT lesions are heterogeneous, i.e., within the same lesion areas of simple, atypical hyperplasia, CIS and invasive carcinoma are seen, these data signify the validity of using these gene products as markers for breast cancer susceptibility and progression.

Immunostaining of lesions for p53 reactivity showed positive reactivity to DO1, a p53 antibody that is reactive to both wild type and mutant p53. However, we did not observe significant increase in p53 immunoreactivity between regions exhibiting atypical hyperplasia and frank invasive carcinoma. We have not been successful in demonstrating immunoreactivity to ER antibody in MCF10AT1 lesions.

Data from these studies, and results obtained from similar analysis of human breast specimens of known pathological grades are being compiled for a manuscript.

Establishment of an in vitro system that recapitulates in vivo aspects of growth and proliferation.

Three-dimensional growth in Matrigel-coated slides: In year 3 of our study we had initiated experiments to study the properties of cells (referred to as EIII8) derived from estrogen-supplemented MCF10AT1 lesions on reconstituted basement membrane. When plated on Matrigel, EIII8 cells exhibit a remarkable ability to polarize and reorganize. Within 12 h of plating, the cells complete organization into structures resembling ducts. While control cultures exhibit organized 3-dimensional growth, they lack the pronounced tube thickening and extremely proliferative papillary bridge forming capacity observed in estrogen-exposed cultures. Inclusion of the antiestrogen, ICI 182,780, in cultures results in thinning of the tubes, reduction in papillary bridge formation, and thus formation of new ducts (Fig. 1). However, unlike ICI 182,780, addition of tamoxifen to estrogen-exposed cultures not only inhibit proliferation but also promote differentiation as evidenced by end bud formation (data not shown).

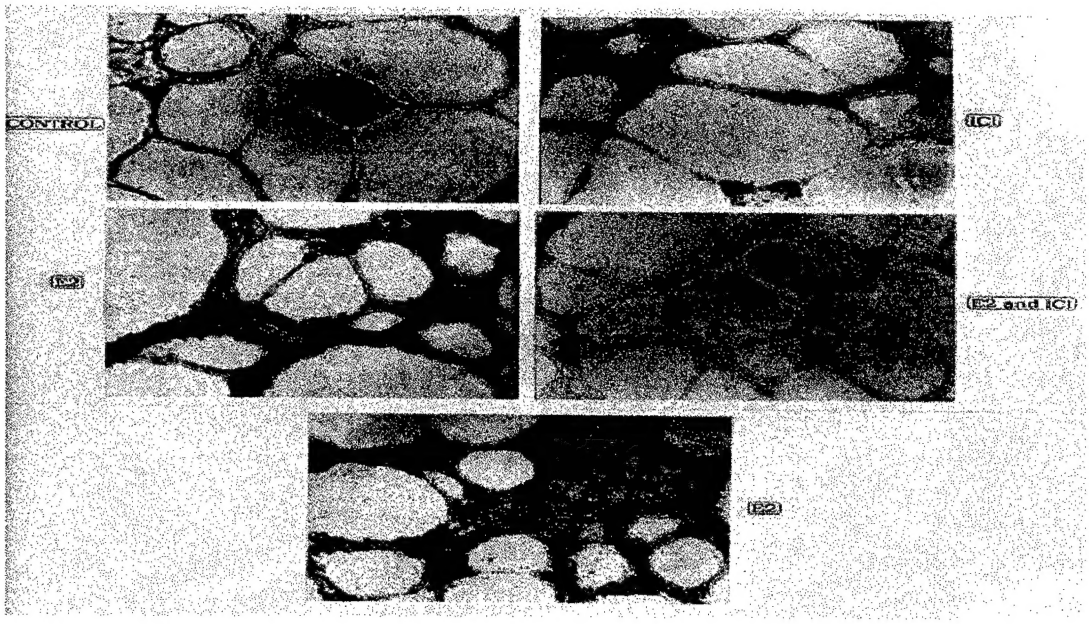


Fig. 1. Effect of estrogen and antiestrogens on 3-dimensional growth of MCF10AT1-lesion derived cells, EIII8, in Matrigel. Note the organization of cells into ductular structures. In the presence of E_2, note that the tubes are multilayered (higher contrast); there are also several new papillary bridges and new ducts. Addition of ICI 184,780 to these cultures causes inhibition of the estrogen-induced effects on ductal growth and proliferation.

Cell Proliferation Assays for determining the effects of estrogen and antiestrogen on 3-dimensional growth of EIII8 cells.

1. MTS assay: 10^5 cells/well were plated in triplicate in Matrigel-coated 8-well chamber slides, and 24 h later the cultures were treated with vehicle (0.01% ethanol), estradiol (E_2, 10^{-10}, 10^{-9} and 10^{-8}M), or 10^{-9}M E_2 plus 10^{-6} M tamoxifen or 10^{-9}M E_2 plus 10^{-7} M tamoxifen. Cultures were fed every other day and proliferation was monitored 5 days later by MTS assay. For measurement by MTS assay, MTS was added to a final concentration of 0.4 mg/ml per well and incubated for 3 h. Media from cultures were removed, centrifuged at 1000xg, and absorbance of supernatants measured at 490 nm.

2. Validation of data from MTS assay by cell counting: Following removal of spent media from the above cultures for MTS assay, dispase was added to cultures to digest Matrigel. Digestion with dispase resulted in complete solubilization of Matrigel leaving the tubular structures intact and organized. These were rinsed in serum free media, trypsinized to obtain single cells and counted. Cell counts were compared with those obtained from the MTS assays.

Results: Results of cell proliferation assays by MTS and direct cell counting were found to be in agreement (Figs. 2 and 3). By both assays, we found the 3-dimensional cultures to be very proliferative. Addition of estradiol did not significantly enhance cell number, although phase contrast microscopy revealed a pronounced thickening of tubes. Addition of tamoxifen in presence of estrogen caused a significant inhibition of cell growth (~50% inhibition, p=0.001) whereas tamoxifen alone did not significantly alter cell growth during the 5 day assay period. The increased rates of proliferation observed in control cultures does not appear to arise from the presence of contaminating estrogen in culture media since tamoxifen alone is unable to significantly inhibit cell proliferation.

Fig. 2. Cell proliferation by MTS assay. Cells were plated on Matrigel and exposed to vehicle (control), tamoxifen, estradiol or a combination of estradiol and tamoxifen at indicated concentrations for 5 days. Absorbance at 490 nm was measured in the spent culture media following color development with MTS reagent as described above..

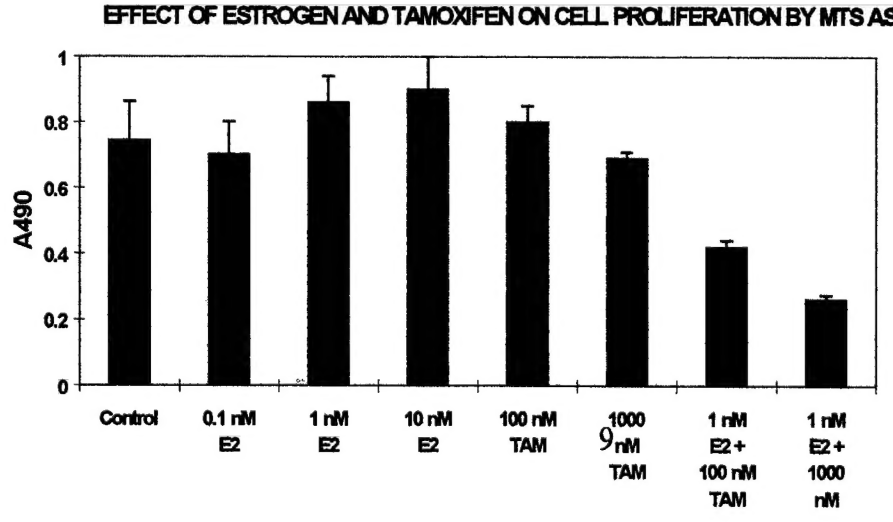
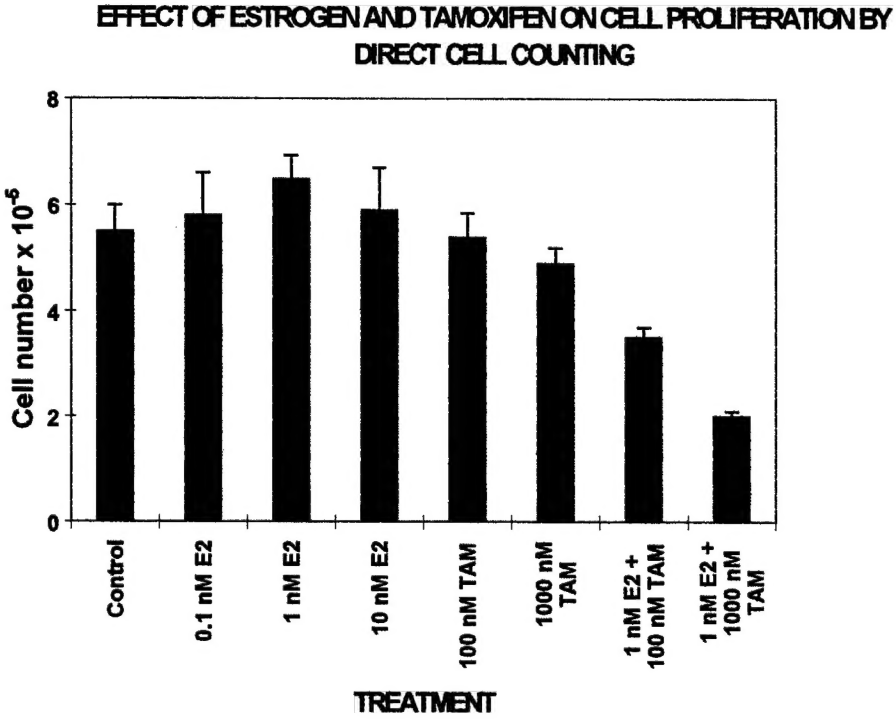


Fig. 3. Cell proliferation by direct cell counting. Cells were plated on Matrigel and exposed to vehicle (control), tamoxifen, estradiol or a combination of estradiol and tamoxifen at indicated concentrations for 5 days. Cultures were treated with dispase to digest Matrigel, trypsinized and counted.Values are mean ± S.E.Values are average of three independent experiments in triplicate.



Morphological analysis of 3-D structures:

Cultures of EIII8 on Matrigel exposed to vehicle (control) or 1 nM estradiol for 24, 48 and 72 h were fixed in buffered formalin and embedded in paraffin. Sections were analyzed for morphology (Hemotoxylin-eosin (H&E) staining; Figs. 4 and 5), and expression of epithelial and mammary markers for differentiation, viz., cytokeratin 18, muc-1 (mucin) and HMFG-1 (human milk fat globulin). Histological analysis of *in vitro* cultures showed recapitulation of lesions derived from *in vivo* experiments (8), viz., the presence of organized ductular structures with multilayered epithelia (Figs. 4 and 5) in both control and estrogen exposed cultures, and immunoreactivity to cytokeratin 18, muc-1 and HMFG-1 (markers of glandular epithelia; Figs. 6 and 7).Analysis of H&E stained sections revealed high proliferative activity as evidenced by presence of several mitotic nuclei in both control and estrogen treated cultures at 24, 48 and 72 h; however, sections recovered from estrogen-treated cultures at all time points revealed the presence of significantly larger number of cells (Compare Figs. 4 and 5). Thus, we are not only able to reproduce *in vitro* morphological and histological characteristics of atypia, an important transitional event in conversion to invasive carcinoma, but also are able to reduce the time required for development

and progression of preneoplastic ducts from 10 weeks *in vivo* to ~5 days *in vitro*. It must be noted that formation of similar three dimensional ductular elements *in vitro* is not a property inherent to most cell systems. Similar culture of tumorigenic MCF-7 cells in Matrigel produce balls of cells that do not differentiate or organize into ducts, and fail to form papillary bridges (data not shown).

Fig. 4. Analysis of H&E sections derived from formalin-fixed paraffin embedded 3-dimensional cultures of control EIII8 cells at 24, 48 and 72 h on Matrigel. Note the presence of multilayered epithelia as early as 48 h of culture. Top row pictures represent 10X magnification of cultures at 24 and 72 h, whereas bottom row indicate 20X magnification of cultures at 48 and 72 h.

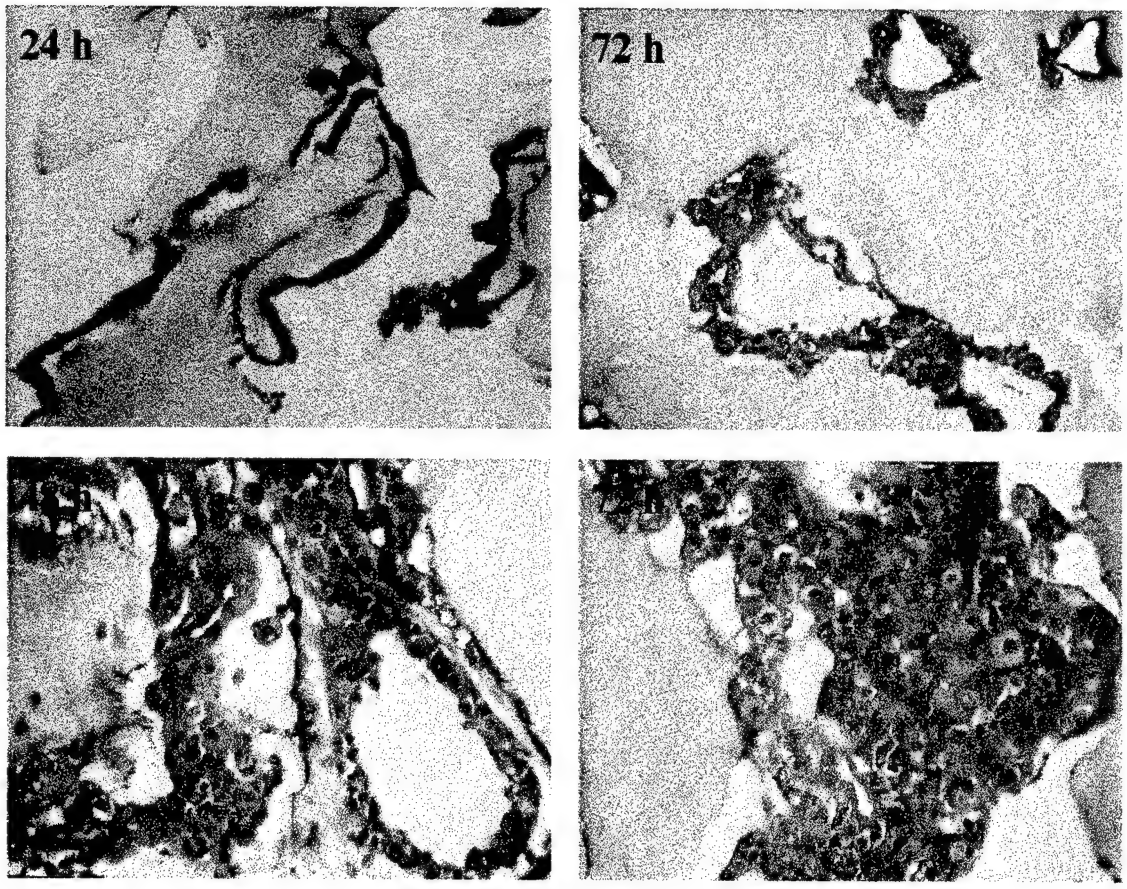


Fig. 5. Analysis of H&E sections derived from formalin-fixed paraffin embedded 3-dimensional cultures of estrogen-exposed (1 nM estradiol) EIII8 cells at 24, 48 and 72 h on Matrigel.Top row pictures represent 10X magnification of cultures at 24 and 72 h, whereas bottom row indicate 20X magnification of cultures at 48 and 72 h. Note the quantitative difference in extent of epithelial cell growth at all time points between control (Fig. 4) and estrogen-treated (Fig. 5) cultures, a situation remarkably similar to that observed *in vivo* in estrogen-supplemented mice.

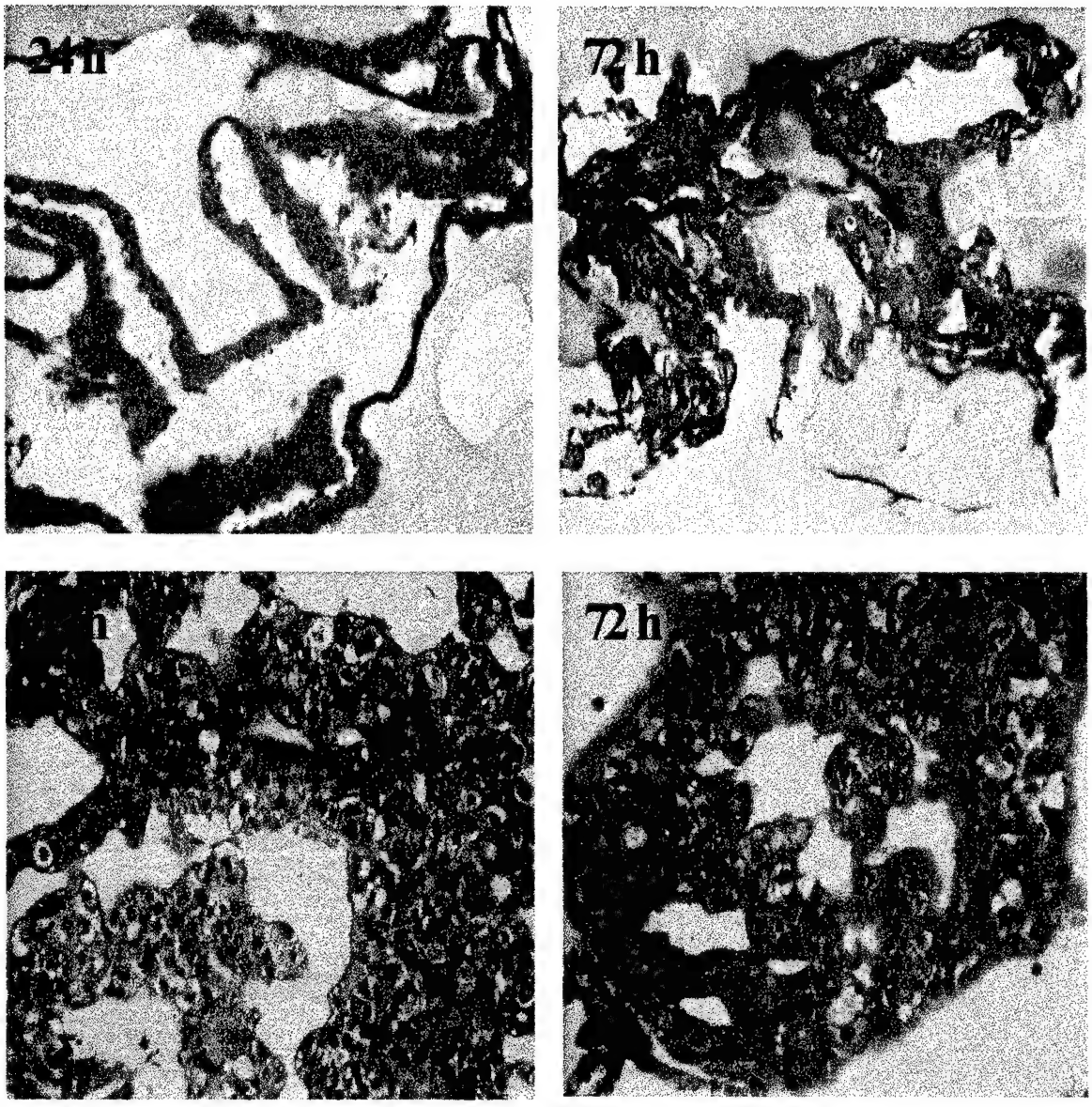
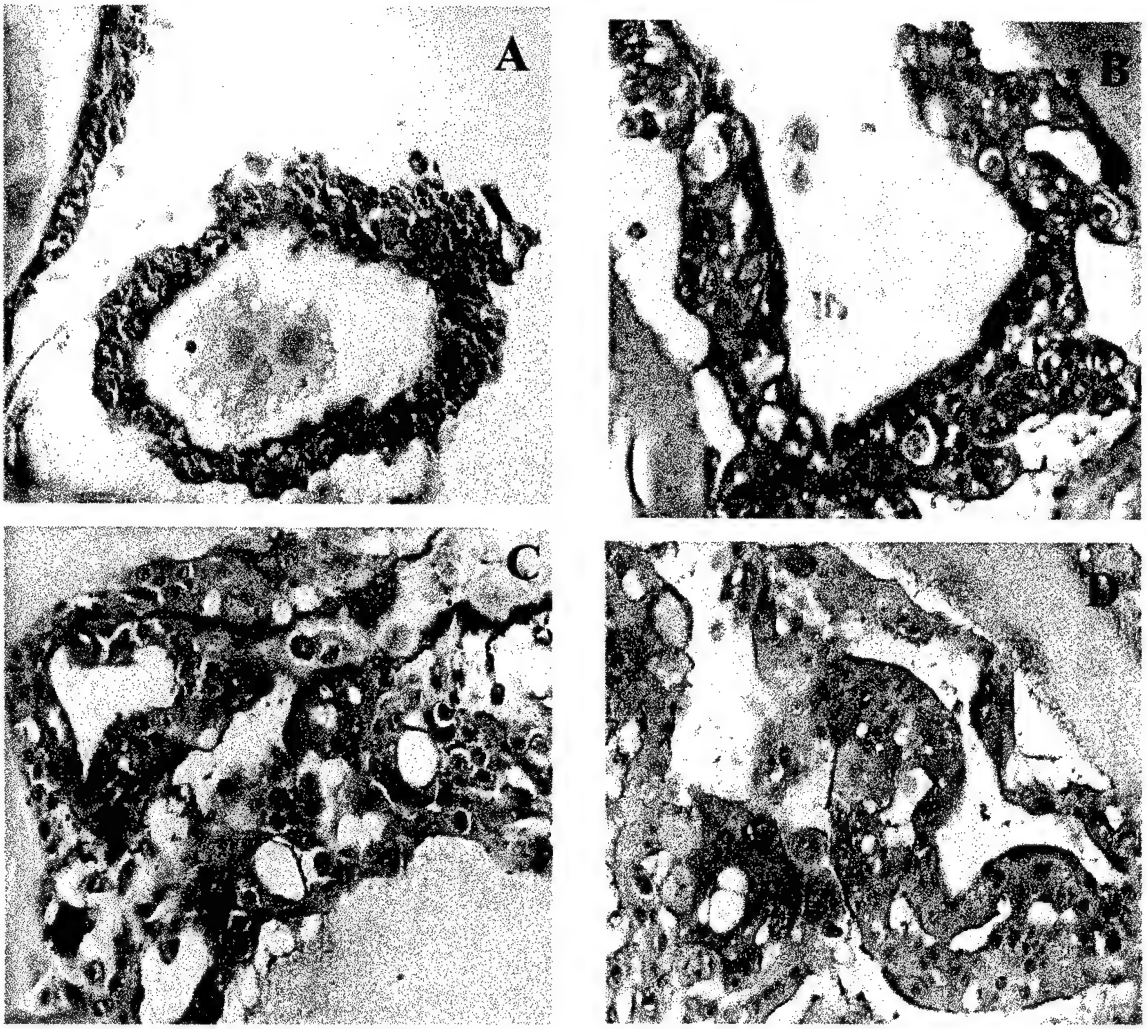


Fig. 6. Immunohistochemical staining of sections derived from 3-dimensional cultures of EIII8 cells on Matrigel. Panels A and B represent staining with cytokeratin 18 antibody, magnifications 10X and 25X, respectively. Note presence of strong immunoreactivity in regions resembling lumen. Panels C and D represent reactivity to anti-HMFG-1 (human milk fat globulin) antibody. Reactivity to anti-HMFG-1 antibody indicates presence of glandular mammary epithelium, a marker for differentiation. Panels C and D represent 25X magnifications.



Since standardization of protocols for paraffin embedding, sectioning and staining of *in vitro* cultures in an intact form consumed enormous amount of time, we were not able to complete staining for bcl-2, erbB-2, cyclin D1, p53 and pS2 on these cultures. This part of work is in progress and is expected to be completed within the next 3 mos.

In vivo effects of tamoxifen on growth and progression of estrogen-induced MCF10AT1 lesions.

Since we have demonstrated effects of estrogen on growth and progression of preneoplastic ducts arising from MCF10AT1 cells (8), it is important to evaluate the effects of tamoxifen, an antiestrogen not only being used for treatment of breast cancer but also as a chemoprevention agent. To determine the effects of tamoxifen on growth and progression of MCF10AT1 and EIII8 cells from the orthotopic site, a total of 10^7 MCF10AT1 or EIII8 cells suspended in Matrigel (Collaborative Research) were injected subcutaneously into the mammary fat pad (orthotopic site) of ovariectomized female nude mice that received subcutaneous implants of estradiol (1.7 mg/90 day release, 10 mice), tamoxifen (5 mg/90 day release, 10 mice) or estradiol plus tamoxifen pellets (10 mice). Control mice received similar implantation of placebo pellets (6 mice). This experiment is still in progress. Animals are being observed twice a week and palpated once a week for lesion formation at the injection site beginning 5 weeks after injection of cells. Mice will be sacrificed at 10 weeks after injection by cervical dislocation. National Institutes of Health guidelines for proper and humane use of animals are being observed. Tissues from the injection sites will be removed, lesions weighed and portions of each fixed in neutral buffered formalin and embedded in paraffin for histological examination. Histological grading of lesions will be done as described previously (1-3); grade 0, simple epithelium; 1, mild hyperplasia; 2, moderate hyperplasia; 3, atypical hyperplasia; 4, carcinoma *in situ*; 5, invasive carcinoma. Each lesion is graded according to the most advanced (deviant from normal) morphological pattern observed within it. All lesions will be examined for histological grading and expression of breast cancer susceptibility markers as desrcribed before. Portions of lesions derived from tamoxifen only or estrogen plus tamoxifen supplemented animals will be digested enzymatically as desribed previously to establish cell lines which will be used for evaluation of sensitivity/resistance to tamoxifen.

Since tamoxifen is being used in clinical trials as a chemoprevention agent, it is important to evaluate its effects on normal mammary gland morphology and function. In order to do this, we will remove mammary glands that did not receive injection of cells from control, tamoxifen only, estrogen only and estrogen plus tamoxifen-supplemented groups, fixed similarly and analyzed for morphology (H and E staining) and function (immunohistochemical staining for mucin, lipid, cytokeratins, TUNEL assay for apoptosis, and Ki67 for proliferative index).

Results of these experiments will allow us to dissect mechanisms of action of tamoxifen both as a chemotherapeutic and chemoprevention agent, viz., whether tamoxifen can block, prevent or reduce growth and progression of estrogen-responsive MCF10AT cells, and if so the specific steps in morphologic sequence of progression affected by tamoxifen. These experiment are already underway. We expect completion of this study to take 6 mos.

REFERENCES

1. Miller, F.R., Soule H.D., Tait, L., Pauley R.J., Wolman S.R., Dawson, P.J., Heppner, G.H (1993). Xenograft model of human proliferative breast disease. J. Nat. Cancer Inst. 85:1725-1732.
2. Dawson PJ, Wolman SR, Tait L, Heppner GH and FR Miller. MCF10AT: A model for

the evolution of cancer from proliferative breast disease. Am. J Pathol 148:313-319, 1996.
3. Huang Y, Ray S, Reed JC, Ibrado AM, Tand C, Nawabi A, and Bhalla K. Estrogen increases intracellular p26Bcl-2 to Bax ratios and inhibits taxol-induced apoptosis of human breast cancer cells. Breast Cancer Res Treat 42:73-81, 1997.
4. Visscher DW, Sarkar F, Tabaczka P and Crissman J. Clinicopathologic analysis of bcl-2 immunostaining in breast carcinoma. Mod Pathol 9:642-646, 1996.
5. Neuman E, Ladha MH, Lin N, Upton TM, Miller SJ, DiRenzo J, Pestell RG, Hinds PW, Dowdy SF, Brown SF and Ewen ME. Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. Mol Cell Biol 17:5338-5347, 1997.
6. Parkes HC Lillycrop K, Howell A and London UK. C-erbB2 mRNA expression in human breast tumors: comparison with c-erbB2 DNA amplification and correlation with prognosis. Br J Cancer 61:39-45, 1990.
7. Ioakim-Liossi A, Karakitsos P, Markopoulos C, Aroni K, Delivelioti K, Gogas J and Kyrkou K. Expression of pS2 protein and estrogen and progesterone receptor status in breast cancer. Acta Cytol 41:713-716, 1997.
8. Direct effect of estrogen on sequence of progression of human preneoplastic breast disease. P.V.M. Shekhar, P. Nangia-Makker, S.R. Wolman, L. Tait, G.H. Heppner and D.W. Visscher. Amer. J Pathol, 152: 1129-1132, 1998.

WORK ACCOMPLISHED IN YEAR FOUR AT UNIVERSITY OF NEBRASKA.
J.K. Christman, M. L. Chen, S.K. Kurumboor and P. Z. Xu.

1. Genomic Instability:

We have continued our studies of genomic instability using frequency of spontaneous appearance of drug (PALA) resistant variants as a marker (1). As noted in our last report, our data support the hypothesis that 'genomic instability' is inversely related to passage number through nude mice and unrelated to the phenotype of the lesion from which the lines are derived.

Our goals were: 1. To determine the possible cause for diminished ability of MCF 1OAT cells to generate PALA resistant colonies after serial xenograft passage. We examined the possibility that cells with a more stable genotype are selected during the initial stage of culturing from xenograft lesion. MCFlOAT3b tumors grown in nude/beige mice were established in culture after dissociation with collagenase and hyaluronidase(2). Thirty single cell clones were grown out to sufficient numbers for freezing and further study. To date we have tested 15 randomly selected clones for genomic stability after four subcultures and all had a frequency of PALA resistance at 9 x LD50 that was less than 1×10^{7}, i.e., no resistant

colonies were observed in 10 cultures of 2 x 10^6 cells. This confirms our results with lines supplied by Dr. Fred Miller (MCF 1 OAT4CCC, 4D, 4000 and 4J)(3).

Although the study is still ongoing, seven clones that have undergone 30 passages have been retested and have frequencies of PALA resistance in the range of 2 x 1 o 6 to 1 x 10 5. This indicates that the majority cells isolated from a fourth transplant generation tumor have a stable genome but do not differ from the parental MCF 1 OA and MCF 1 OAT cells in their capacity to acquire genomic instability with time in culture.

It should be noted that the study from which these MCF 1OAT3b lesions were obtained was separately funded by a non overlapping Eppley Institute, American Cancer Society Institutional Grant to JC. The lesions were not required for interpretation of the results of the ACS study.

2) To determine the nature of the amplification of the CAD gene MCF1OAT4 cells selected at 6 x LD50. A major problem incurred was the preparation of metaphase spreads for cytogenetic analysis of CAD gene amplification in the initially resistant cells. However, FACS analysis indicated that the cells were hypertetraploid. (Figure 1 shows a representative experiment). This suggests that amplification of CAD gene occurs in the PALA resistant cells through unequal duplication of chromosomes including chromosome 2 rather than through tandem array amplification of CAD genes.

2. Analysis of P53 in MCF1OA and MCFlOATn cells by immunostaining.

In year three, as previously reported, we completed analysis of P53 protein both by FACS and immunostaining. We proposed to carry out an analysis of the distribution of conformationally altered and native P53 in areas of MCF1OAT lesions at morphologically distinct stages of progression with those seen in cycling and stationary cell cultures. It was proposed to use lesions from both estrogen supplemented and normal female hosts. Since the current grant provided neither budget nor permission from the Department of the Army to carry out animal studies for this proposal at UNMC, and since no such lesions were provided by Dr. Shekhar, these studies could not be carried out.

Analysis of p53 expression in lesions was carried out by Dr. Shekhar in collaboration with Dr. Dan Visscher, an experienced breast pathologist at Department of Pathology, Wayne State University School of Medicine.

3. Role of DNA Methylation Changes in ER expression:

A complete analysis of methylation in the proximal promoter region and exon was completed using bisulfite sequencing and a summary reported at the end of the third year. Our results suggested that unlike breast tumor cells (4), silencing of ER expression is not accompanied by extensive exon 1 methylation in the near normal MCF 1 OA lines and that its reactivation in

MCF 1 OAT cells does not depend on complete loss of methylation in exon 1. Our goal for year four was to confirm this by determining whether 5 azaC treatment would increases ER production in MCF1OAT3B cells as would be predicted if loss of the variable methylation in exon 1 is associated with increased expression of these cells. Cells were treated for three days with 1 or 3 I1M of the DNA methyltransferase inhibitor 5 azaC under condition similar to those previously used by us and others (5, 6). Since the cells ceased or slowed growth when this drug concentration was maintained, the cells were allowed to recover for 5 days in the absence of drug and then passed through another exposure of four days. Estrogen receptor (ER) expression was monitored by PCR detection of ER mRNA and immunostaining with counting of ER positive nuclei after each round of treatment. Three separate experiments were carried out. ER mRNA and protein were detected in all cultures (~ 15% of cells had detectable nuclear staining). Although treated cultures always had a slightly higher % of ER positive cells immediately after four days exposure to 5 azaC (17 20% ER+), there was no significant increase in % of ER+ cells or intensity of nuclear staining. This data support our initial conclusion that DNA methylation is not the limiting factor regulating ER expression in MCF 1 OA and MCF 1OAT cells.

REFERENCES

1. Tlsty, T. D. Normal diploid human and rodent cells lack a detectable frequency of gene amplification. Proc. Natl. Acad. Sci. USA, 87: 3132 3136, 1990.

2 Miller, F. R., Soule, H. D., Tait, L., Pauley, R. J., Wolman, S. R., Dawson, P. J., and Heppner, G. H. Xenograft model of progressive human proliferative breast disease. J. Natl. Cancer Inst., 85: 1725 1732, 1993.

3. Kurumboor, S. K., Shekhar, P. V. M., and Christman, J. K. Xenograft passage is associated with decreased frequency of PALA resistant variants in MCF 1 OAneoT cells. Proc. Amer. Asso. Cancer Res., 37: Abstract #222, 1996.

4. Ottaviano, Y. L., Issa, J. P., Parl, F. F., Smith, H. S., Baylin, S. B., and Davidson, N. E. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res., 54: 2552 2555, 1994.

5. Ferguson, A. T., Lapidus, R. G., Baylin, S. B., and Davidson, N. E. Demethylation of the estrogen receptor gene in estrogen receptor negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res., 55: 2279 2283, 1995.

6. Creusot, F., Acs, G., and Christman, J. K. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5 azacytidine and 5 aza 2' deoxycytidine. J. Biol. Chem., 257: 2041 2048, 1982.

Our manuscript "Role of genomic instability in progression of MCF1 OAT xenograft lesions:

A study of drug resistance in human breast epithelial cell lines comprising a xenograft model of proliferative breast disease." was held back pending completion of the proposed cytogenetic studies described above. It is expected to be submitted within the next month.

A second manuscript "DNA methylation and regulation of estrogen receptor expression in MCF 1 OA and MCF 1 OAT cells" is under preparation.

CONCLUSIONS

1. We have established the specificity and validity of specific gene products that can be used as molecular markers for breast cancer progression, viz., bcl-2 specifically associated with simple glandular areas; erbB-2 is associated with regions showing atypical hyperplasia and more advanced grades of progression; whereas cyclin D1 is specifically found in nuclei of cells that are invasive. While progressed areas show a definite increase in p53 immunoreactivity, there is no significant difference in reactivities between grades 3 and 5.

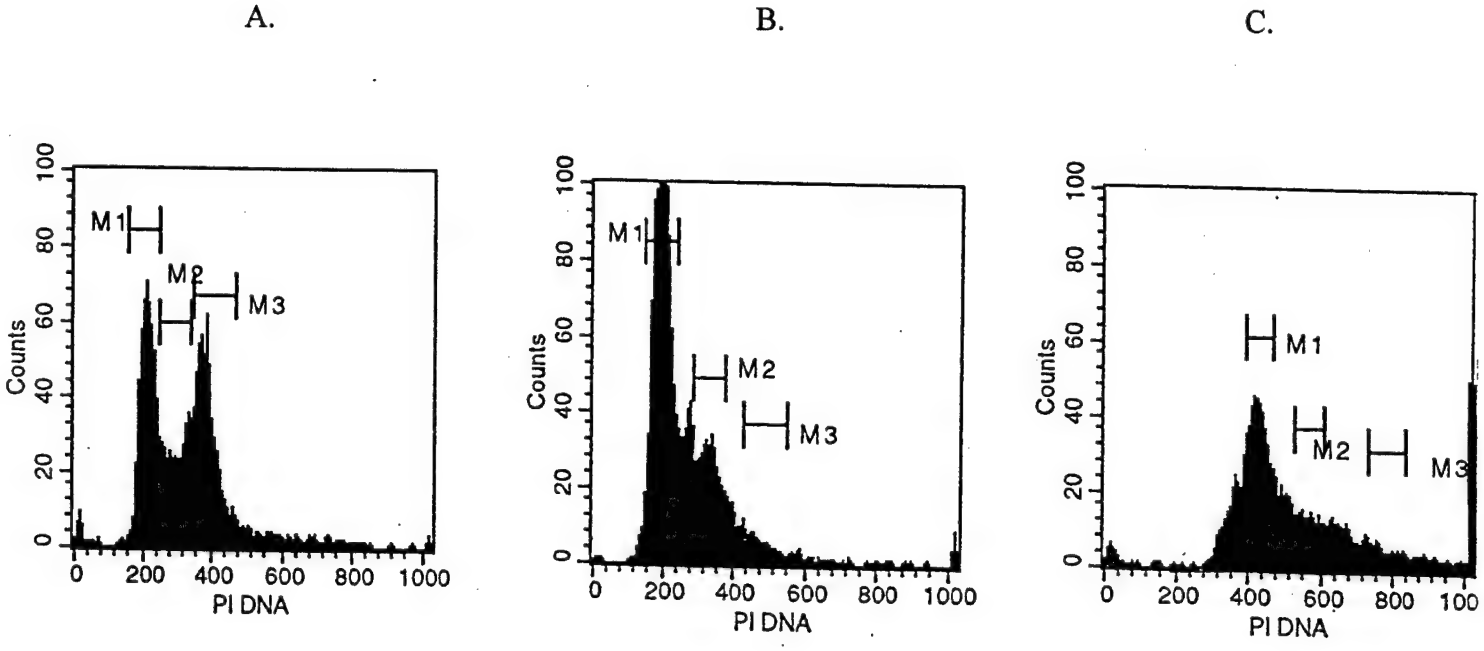
2. We have established an *in vitro* assay system that recapitulates the *in vivo* organization and proliferative capacity of preneoplastic human breast cells. Data from growth assays suggest this system may be useful for defining effects of tamoxifen and other chemotherapeutic and chemoprevention agents on growth, proliferation, differentiation and apoptosis.

3. Mechanisms responsible for transcriptional activation of ER gene do not involve (a) alterations in methylation status of ER gene as reported in other ER positive (MCF-7) and ER negative (MDA-MB 435) cells, or (b) position effect resulting from insertion of exogenous *T24-Ha ras* gene in MCF10AT cells.

EXPERIMENTS TO BE COMPLETED IN 1998-'99

1. Characterization of 3-dimensional *in vitro* cultures for molecular correlates of progression.
2. Effects of tamoxifen on progression of estrogen-responsive MCF10AT cells: chemotherapeutic and chemoprevention aspects.

Figure 7 FACS Analysis of MCF10AT4D Cells



Panel A. Cells from subconfluent culture, no treatment.

Panel B. Cells from confluent culture, no treatment.

Panel C. Cells from culture grown in the presence of 79 μM (6 x LD_{50}) PALA for 2 weeks.

DNA was stained with Propidium Iodide prior to analysis.

American Journal of Pathology, Vol. 152, No. 5, May 1998

Short Communication

Direct Action of Estrogen on Sequence of Progression of Human Preneoplastic Breast Disease

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From the Breast Cancer Program and Metastasis Program,‡ Karmanos Cancer Institute, and the Departments of Pathology† and Internal Medicine,¶ Wayne State University School of Medicine, Detroit, Michigan, and the Department of Pathology,§ Uniformed Services University of the Health Sciences, Bethesda, Maryland*

We have used the MCF10AT xenograft model of human proliferative breast disease to examine the early effects of estradiol exposure on morphological progression of preneoplastic lesions and to define the step(s) in the morphological sequence at which estrogen may act. The effects of estradiol on neoplastic progression of estrogen-receptor-positive MCF10AT cells in the orthotopic site were examined in ovariectomized female nude mice that received subcutaneous administration of implants of 17β-estradiol or placebo pellets. At 10 weeks, histological analysis of the lesions derived from the estrogen-supplemented group revealed that 92% of lesions displayed histological features of atypical hyperplasia, carcinoma *in situ*, or invasive carcinoma, and the remaining 8% exhibited histological features of moderate hyperplasia. These highly proliferative lesions are in marked contrast to the control group in which 60% of samples displayed no evidence of hyperplasia. In contrast with control xenografts, estrogen-exposed xenografts demonstrated extensive areas of papillary growth, adenosis-like areas, prominent host inflammatory infiltration, and angiogenesis. Our results suggest that estrogen exerts a growth-promoting effect on benign or premalignant ductal epithelium by enhancing 1) the frequency of lesion formation, 2) the size of lesions, 3) the speed of transformation from normal/mild hyperplasia to those with atypia, 4) the degree of dysplasia, and 5) angiogenesis. *(Am J Pathol 1998, 152:1129–1132)*

The development of strategies for breast cancer prevention depends on improved understanding of the molecular and cellular events that lead to transformation and neoplastic progression of human breast epithelial cells. Epidemiological and experimental evidence suggests that breast cancer risk is related to the duration of estrogen exposure during puberty, the early postmenarchial period, and the menopausal period.[1,2] The effects of estrogen on the proliferation of target breast cells are believed to be mediated through transactivation of specific genes that are recognized by the estradiol-estrogen receptor (E_2-ER) complex.[3] This process stimulates DNA synthesis, cell division, and production of biologically active proteins, such as pS2, transforming growth factor-α, and epidermal growth factor,[4] which influence cell growth and differentiation. Exposure to estrogen may contribute to mammary carcinogenesis by stimulating proliferation of a clone of precancerous cells or by increasing the chance of spontaneous mutation. Alternatively, estrogen could decrease cell cycle transit time so that a spontaneous mutation becomes fixed before repair. An additional possibility is that estrogen may have a direct genotoxic effect.[5,6] Thus, despite wide agreement that estrogen is involved in the etiology of breast cancer, there is uncertainty as to the precise role of estrogen in the biology of breast cancer induction. Much of this difficulty can be ascribed to the lack of relevant model systems to understand the mechanism of early estrogen action in human mammary tumorigenesis.

In the present study we have used the MCF10AT xenograft model of human proliferative breast disease[7] to 1) examine the influence of estrogen exposure on the morphological progression of preneoplastic lesions and 2) define the step(s) in the morphological sequence at which estrogen may act. Previous studies have shown that MCF10AT cells grow progressively in immunodeficient mice, in which over a period of several months they

Supported by grants from the U.S. Army Medical Research and Materiel Command (DAMD17–94-J-4427) and the National Institutes of Health (CA60881 and CA22453).

Accepted for publication February 6, 1998.

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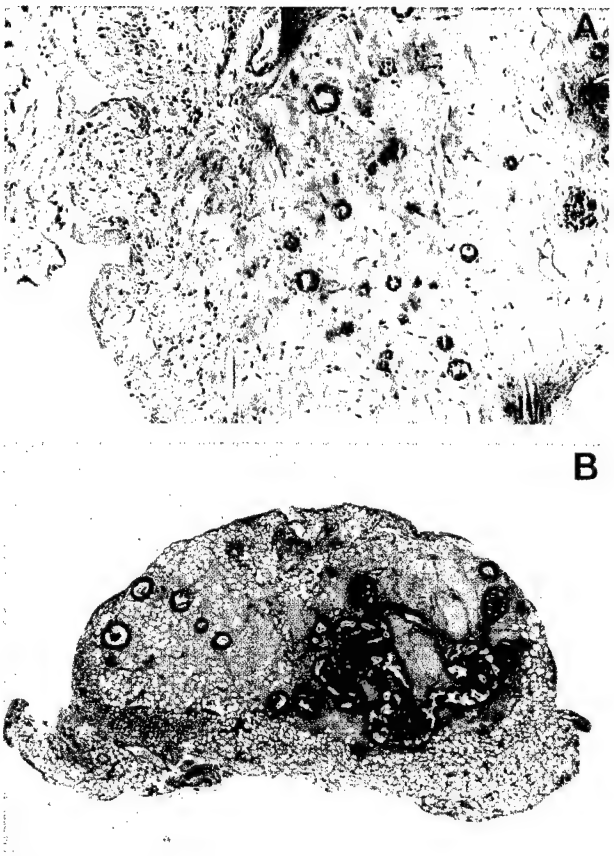

Figure 2. Histological features of MCF10AT1 lesions derived from control and estrogen-supplemented mice. **A:** Control lesion at 70 days of injection. Note that majority of the lesion consists of epithelial ducts that are lined by one layer of epithelial cells. H&E; magnification, ×10. **B:** Lesion from estrogen-treated nude mouse at 35 days af injection of MCF10AT1 cells. Note that the bulk of the lesion is displaying features of grade 3, although few ducts with simple epithelia and some with papillary bridging (grade 2) are still present. H&E; magnification, ×10.

hibit spontaneous gain in expression of functionally active estrogen receptor[9,10]; however, the extent of contribution of the E_2-ER complex to angiogenesis remains to be established.

Table 1. Distribution (%) of Histological Grades by Extent in Estrogen-Exposed MCF10AT1 Xenografts

	Distribution (%)				
Case	Grade 0/1	Grade 2	Grade 3	Grade 4	Grade 5
1	0	50	0	50	0
2	0	60	20	20	0
3	0	100	0	0	0
4	10	30	30	30	0
5	20	40	40	0	0
6	10	20	20	30	20
7	20	50	30	0	0
8	20	40	20	20	0
9	10	60	30	0	0
10	0	30	40	30	0
11	0	0	0	0	100
12	10	60	30	0	0
13	20	30	50	0	0

Grade 0/1, simple/mild hyperplasia; grade 2, moderate hyperplasia; grade 3, atypical hyperplasia; grade 4, carcinoma in situ; grade 5, invasive carcinoma.

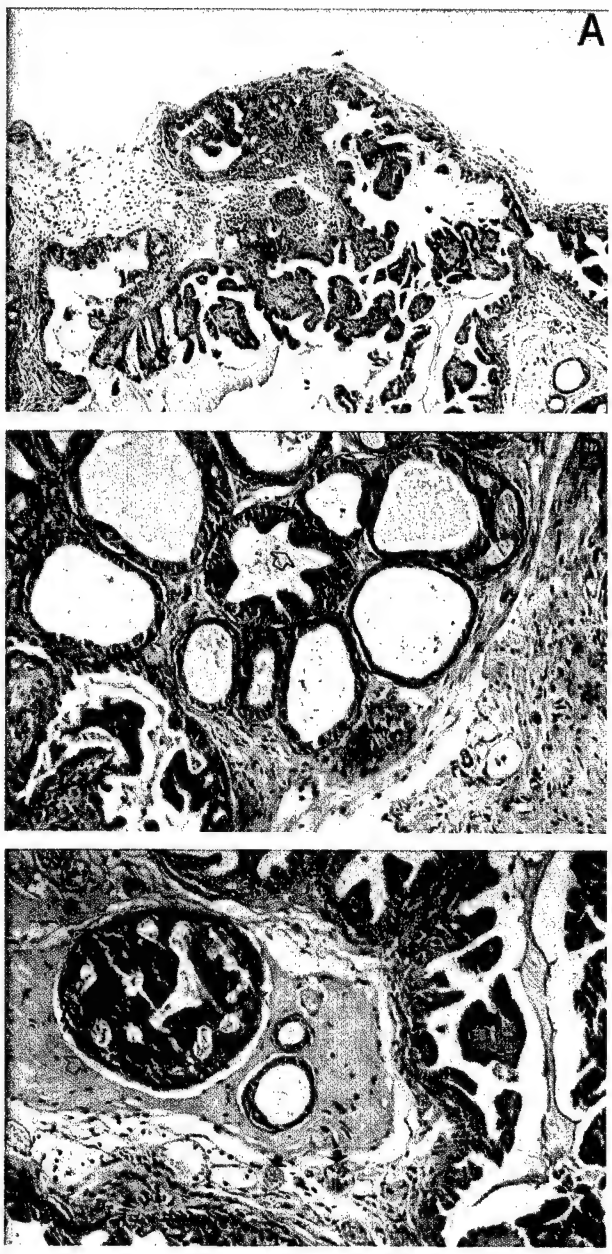

Figure 3. Morphological alterations induced by exposure of MCF10AT1 lesions to 17β-estradiol. **A:** Florid papillary hyperplasia within a cystically dilated space. Cystic dilation was characteristic of estrogen-treated lesions. H&E; magnification, ×50. **B:** Glandular adenosis. Note that the central gland demonstrates mild hyperplasia that is indicated by an **arrow**. H&E; magnification, ×100. **C:** Angiogenesis. Note the presence of numerous blood vessels (indicated by **solid arrow**) in a lesion that shows an extended duct (**open arrow**) at grade 4 and surrounding areas of papillary hyperplasia. H&E; magnification, ×100.

Previous studies have explored the MCF10AT model for human proliferative breast disease and development. However, in these studies there was 1) nonuniformity of progression to cancer and 2) a prolonged delay in most hosts, and 3) the overall rate of cancer induction was ~30%.[8] In contrast, in the presence of estrogen, this model exhibits premalignant and malignant lesions at high frequency in a relatively short time frame. The demonstration of a direct action of estrogen on the sequence of neoplastic progression of ER-positive MCF10AT cells

Transcriptional activation of functional endogenous estrogen receptor gene expression in MCF10AT cells: A model for early breast cancer

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Abstract. Utilizing the MCF10AT xenograft model for progression of human proliferative breast disease, we detected expression of the endogenous estrogen receptor (ER) gene only in MCF10AneoT and cells of the MCF10AT system, all of which stably express a transfected mutated T24 *Ha-ras* gene. ER transcripts were undetectable in the parental MCF10A cells and in MCF10A cells transfected with normal *c-Ha-ras* or vector. ER transcripts expressed in MCF10AT cells contain a normal full-length ER coding region and direct synthesis of a normally sized ER protein. The protein is functional based on its ability to mediate estradiol (E_2)-induced increases of transcription from both endogenous and exogenous E_2-regulated genes. Transcriptional activation of the endogenous ER gene does not appear to be related to a change in methylation status of the gene since a diagnostic CpG site in exon 1 that is methylated in ER-negative breast tumors and completely unmethylated in ER-positive breast tumors is hypomethylated to the same extent in ER-negative MCF10A cells and ER-positive MCF10AT cells. E_2 increased both the number and size of soft-agar colonies formed by MCF10AT3c cells, a line from a third generation MCF10AT xenograft lesion. This suggests that xenograft passage has selected for growth regulatory pathways that are E_2-responsive and that identification of these pathways and their role in progression will aid in determining how E_2 acts to increase risk of breast cancer.

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Key words: MCF10A, xenograft model, estrogen receptor, T24 *Ha-ras*, preneoplasia, breast cancer progression

Introduction

Breast cancer is one of the most common malignancy among women. Although very little is known about the molecular events underlying the development of mammary tumors in humans, much evidence indicates that the presence of ER is generally a favorable prognostic marker because ER-positive tumor cells are usually more differentiated and have lower metastatic potential than ER-negative tumor cells. Only 6-10% of normal human breast epithelial cells are ER-positive (1,2), yet more than 60% of primary human breast tumors are ER-positive and initially depend on E_2 for growth (3-5).

We have utilized the MCF10AT xenograft model of early human breast cancer progression (6). MCF10AneoT cells are a T24 *Ha-ras* transformed line derived from MCF10A preneoplastic human breast epithelial cells (7). MCF10A cells form lesions in *nu/nu* beige mice that regress within five weeks, in contrast, MCF10AneoT cells form persistent lesions (6). MCF10AneoT and lines derived by alternating *in vivo* transplantation and *in vitro* culture (MCF10ATn) are collectively known as the MCF10AT system (8). The lesions formed by lines of the MCF10AT system are composed of a heterogeneous spectrum of ductular tissues with a range of morphology that includes mild hyperplasia, atypical hyperplasia, carcinoma *in situ* (CIS), moderately differentiated carcinoma, and undifferentiated carcinoma, as well as histologically normal ducts (6,8). Thus, the MCF10AT system provides a transplantable, xenograft model of human proliferative breast disease with proven neoplastic potential. Approximately 62% of lesions produced in mice injected with MCF10AneoT cells are comprised of simple to mildly hyperplastic epithelia while the remaining 38% contain elements of invasive carcinoma irrespective of whether the host is male or female (8). In contrast, MCF10AT1 cells, a line derived from an MCF10AneoT lesion containing elements of squamous carcinoma form lesions with a wider range of morphology than those formed by MCF10AneoT (8) but, to date, have formed lesions with invasive carcinoma only in female mice (F. Miller, unpublished data). This suggested to

us that ER-mediated processes might play a role in MCF10AT progression after initial selection for the capacity to form persistent lesions. However, MCF10A cells, from which the cell lines of the MCF10AT system are derived, are ER-negative at both the protein and mRNA level (9,10). Here, we present evidence that the endogenous ER genes in MCF10A cells have been activated in MCF10AT cells leading to expression of functional ER protein. The implications of this event for progression of MCF10AT system xenografts are discussed.

Materials and methods

Cell lines and cell culture. These studies utilized parental MCF10A, MCF10Aneo (vector transfected), MCF10AneoN (normal *Ha-ras*-transfected), and the following lines from the MCF10AT system: MCF10AneoT, MCF10AT1, 2b and 3c. Cells from all of these lines were maintained in phenol red-free DMEM/F-12 medium with 0.1 µg/ml cholera toxin, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 0.02 µg/ml epidermal growth factor, 100 i.u./ml penicillin, 100 µg/ml streptomycin, and 2.5% horse serum (DMEM/F12^{sup}). Charcoal-stripped serum was not used since it reduces the proliferative capacity and/or viability of MCF10A cells, possibly due to removal of essential growth factors (11). The only sera used routinely were those which were unable to support growth of the ER-positive cell line, MCF-7, indicating absence of biologically significant levels of E_2 or other estrogenic compounds. MCF-7 cells, which were used as a positive control for ER expression, were grown in Eagle's minimal essential medium with Hank's balanced salt solution, 2.5 mM L-glutamine, and 25 mM HEPES, supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 5 µg/ml gentamycin and 5% donor calf serum. T47D cells were used as a positive control for PgR expression and were grown in Eagle's MEM supplemented with 0.1 mM non-essential amino acids, 2 mM L-glutamine, 5% fetal calf serum and 6 ng/ml of insulin. To induce PgR, MCF10AneoT and MCF10AT3c cells were treated for 3 days with 1 nM E_2 dissolved in ethanol (0.01% final concentration). Controls were treated with ethanol alone.

Reverse transcriptase (RT)/PCR. Two µg of RNA were denatured at 65°C for 5 min and reverse transcribed in the presence of 1 mM dATP, 1 mM dCTP, 1 mM dTTP, 1 mM dGTP, 5 mM dithiothreitol (DTT), 1 unit/µl RNase inhibitor, 20 µM random primers (Pharmacia, Piscataway, NJ), reverse transcriptase buffer and 5 units/µl reverse transcriptase (Seikagaku, Japan) for 20 min at room temperature and 1 h at 42°C followed by 10 min at 95°C. PCR was performed using 0.5 µM concentration of each primer and 0.5 unit Taq polymerase (Promega, Madison, WI) in a total volume of 100 µl. PCR products were further characterized by restriction mapping, Southern blot analysis and DNA sequencing.

Detection of ER mRNA expression. Total RNA was reverse transcribed as described above. An oligonucleotide primer set (sense 5'-GCCCGCGGCCACGGACCATGACCAT-3', and antisense, 5'-TGACCATCTGGTCGGCCGTC-3') flanking the human ER cDNA sequence from base -17 to 950 was used for amplification by PCR yielding a 967 bp fragment that includes exons 1-6 of ER (12). The identity of the 967 bp ER cDNA fragment amplified from MCF10AneoT and derivatives was confirmed by Southern blot hybridization analysis with a full-length human ER cDNA probe. Full length ER cDNAs were amplified from MCF10AneoT and MCF10AT3c by RT-PCR using primer sets flanking base -17 to 1735 (12) and cloned into TA-cloning vector (Invitrogen, San Diego, CA). Both strands were sequenced using ER specific primers.

Analysis of c-Ha-ras mRNA expression. PCR amplifications of reverse transcribed *Ha-ras* mRNAs were carried out with an oligonucleotide primer set flanking the *c-Ha-ras* sequence from base 18-556 of the human *Ha-ras* cDNA (sense, 5'-CGA TGACGGAATATAAGCTGGTGGTGGT-3', and antisense, 5'-GGGGCCACTCTCATCAGGAGGGTTCAGCTT-3'). This includes bases 1661-3317 of the human *c-Ha-ras* gene (13). PCR products were cleaved with *Nae*I. All single base *ras*-activating substitutions at codon 12 eliminate a *Nae*I site (GCC/GGC) and give a novel 179 bp fragment under these conditions. *Nae*I-restricted cDNAs were analyzed by electrophoresis through 2% agarose gels.

Analysis of ER expression

Immunodetection of ER. MCF10Aneo, MCF10AneoT, MCF10AT3c and MCF-7 cells that had been grown to near confluence were rinsed in phosphate buffered saline (PBS), harvested by trypsinization, pelleted and resuspended in a high salt buffer containing 10 mM $NaMoO_4$, 10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT and 0.6 M KCl, pH 7.5. Lysates were centrifuged at 100,000 x g for 30 min and the supernatant (cytosolic) fractions incubated with human anti-ER monoclonal antibody, D547 (gift from Dr Geoffrey Greene), followed by the addition of Protein A Sepharose. Bound ER was extracted from the Sepharose resin by boiling in Laemmli buffer and separated on a 7.5% sodium dodecyl sulphate (SDS)-polyacrylamide gel (14). Proteins were electroblotted onto Immobilon-P membranes, incubated sequentially with anti-ER monoclonal antibody D547 and ^{125}I-labeled anti-rat IgG. Following four washes with buffer containing 50 mM Tris-HCl/0.3 M NaCl/1% Triton X-100/1 mM phenylmethanesulfonyfluoride (PMSF), pH 7.5, the membrane strips were dried and exposed to Kodak XAR film at -70°C for 1-3 days. The relative amounts of ER expressed were quantitated with a densitometer (Model 300A, Molecular Dynamics, Sunnyvale. CA).

Immunofluorescence microscopy. MCF10AT3c and MCF-7 cells were grown to near confluence on Lab-Tek chamber slides (Nunc, Naperville, IL). The cells were fixed and immunostained with rat monoclonal antibody to human ER. H222, following directions provided by the supplier of the ER monoclonal antibody. Controls were similarly treated with normal rat IgG or buffer alone. The cells were washed with PBS and incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Zymed Laboratories. Inc., San Francisco, CA) and rinsed with PBS. Slides were viewed with a 63X oil objective using a Nikon Optiphot microscope with epifluorescence.

Fluorescence in situ hybridization (FISH). Slides of metaphase cells were prepared and analyzed by C.B. Talmadge in the

University of Nebraska Medical Center Imaging System Core Facility essentially as described in ref. 15. pHO6T1 (16), the plasmid used to introduce mutated (T24) *c-Ha-ras* gene into MCF10A cells (7) was labeled with digoxigenin-11-dUTP (Boehringer Mannheim) for use as probe. Probes were detected with fluorescein isothiocyanate-labeled sheep anti-digoxigenin (10 µg/ml; Boehringer Mannheim). The slides were mounted in fluorescence antifade solution pH 11.0 containing propidium iodide (Vector Laboratories). Images were obtained with a Nikon microphot FXA microscope coupled to a cooled charge-coupled device camera controlled by a computer with Oncor Image V1.6 FISH application software.

Determination of endogenous ER function

Reporter plasmid construct. Construction of the ERETKCAT vector, JA-12, is described in ref. 17. JA-12 contains tandem EREs (one consensus and one mutated) between the *Xba*I site and the TATA box of p 37TKCAT (18).

Cell culture and transfection. MCF10AneoT cells (500,000/60 mm dish) were plated in phenol red-free DMEM/F12^{sup} medium and maintained for 18 h prior to transfection with 10 µg of JA-12 plasmid DNA using the calcium phosphate method (19). Six hours after transfection, cells were shocked using a 3 min incubation with 15% glycerol, washed and cultured in phenol red-free DMEM/F12^{sup} medium containing E_2 and/or ICI 164,384 or vehicle (ethanol) at indicated concentrations for 36-42 h.

Chloramphenicol acetyl transferase (CAT) assay. Transfected cells were lysed with four cycles of freezing and thawing in 0.25 M Tris-HCl, pH 7.5. CAT assays were carried out as described by Gorman *et al* (20) with reaction mixtures containing 100 µg of supernatant protein and 0.1 µCi of ^{14}C-chloramphenicol (40-60 mCi/mmol, ICN Radiochemicals, Irvine, CA). The assay tubes were incubated for 4 h at 37°C. Acetylated chloramphenicol was separated by thin layer chromatography and quantitated by liquid scintillation counting. Protein concentrations were determined by the Bradford method (21). The lacZ reporter plasmid pCH110 (Pharmacia, Piscataway, NJ) was cotransfected in some experiments to monitor transfection efficiency and assayed according to the manufacturer's protocol. ß-galactoside standard and o-nitrophenyl-galactoside were purchased from Boehringer Mannheim (Indianapolis, IN).

Analysis of progesterone receptor (PgR) expression. Cell lysates prepared from MCF10AneoT and MCF10AT3c cells cultured in phenol red-free DMEM/F12^{sup} medium with or without added E_2 (10^{-9} M) were incubated with anti-human PgR mouse monoclonal antibody Ab-1 (Oncogene Science, Cambridge, MA) overnight at 4°C. Immune complexes were pelleted with Protein G Sepharose, solubilized by boiling in SDS-sample buffer and subjected to SDS-PAGE and Western blot analysis as described above. Lysates prepared from the PgR-positive breast cancer cell line, T47D, were treated in the same way and used as controls. Proteins transferred to Immobilon-P (Millipore Corp., Bedford, MA) membranes were incubated sequentially with the same anti-PgR antibody and ^{125}I-labeled goat anti-mouse IgG (ICN Radiochemicals, Irvine, CA). Similar analysis of PgR proteins was also carried out by immunoprecipitation/Western blot analysis using cell extracts prepared from two estrogen-responsive clones of MCF10AT3c cells (clones A and B).

Growth in soft agar. $4x10^4$ MCF10AneoT or MCF10AT3c cells were seeded in 2 ml of 0.3% agar in phenol red-free DMEM/F12^{sup}. This suspension was layered over 1 ml of 0.9% agar medium base layer in 35-mm dishes (Costar) and overlayered with 2 ml of phenol red-free DMEM/F12^{sup} containing 10^{-8} M or 10^{-9} M E_2 or an equivalent concentration of vehicle (ethanol). All dishes were incubated at 37°C in 5% CO_2: 95% O_2 for 4 weeks with twice-weekly media changes. All cultures were examined 24 h after plating and cell aggregates that might bias final results were marked. Plates with more than 10 aggregates were discarded. Colony forming efficiency (CFE) was calculated by dividing the number of colonies larger than 50 µm (sized using a calibrated ocular grid) by the number of cells seeded. Ten microscopic fields were counted to calculate the total number of colonies/well for the whole well; reported values are the average count from triplicate wells. The number of colonies in different size ranges (50-100 µm, 100-200 µm, 200-300 µm and >300 µm) was calculated in the same manner.

Determination of methylation status of ER. Ten µg of purified genomic DNA (22) was digested with *Eco*RI (4 units/µg DNA) and *Not*I (4 units/µg DNA) for 16 h. *Not*I will only cleave DNA at its recognition site (GC/GGCCGC) if the indicated C residue in this site is unmethylated on both strands (23). This means that methylation by mammalian C-5-DNA MTase at either CpG dinucleotide in the *Not*I site will prevent cleavage. The resulting DNA fragments were separated by electrophoresis on 1% agarose gel, blotted and probed with a 0.3 kb *Eco*RI/*Pvu*II fragment of the ER gene obtained from the plasmid POR3 (ATCC-57681). Completeness of cutting was verified by determining that cleavage was complete at a c-*myc* CpG island *Not*I site that is normally unmethylated *in vivo* (24).

Results

Analysis of ER expression in the MCF10AT system. To determine whether ER is expressed in MCF10AT system cells, RT-PCR with ER-specific primers was used to detect ER mRNA expression. A 967 bp ER fragment amplified by PCR was detected in all cells of the MF10AT system examined (Fig. 1, lanes 4-7), whereas this fragment was undetectable in the parental MCF10A cells and its non-lesion forming derivatives, MCF10Aneo and MCF10AneoN (Fig. 1, lanes 1-3). The origin of the 967 bp fragment was confirmed by Southern blot hybridization (Fig. 1) and DNA sequencing. Full length ER cDNAs amplified from MCF10AneoT and MCF10AT3c cells were cloned into TA-cloning vectors (Invitrogen, San Diego, CA) and sequenced to determine whether the coding regions of the endogenous ER gene of MCF10AT cells had undergone any alterations in sequence. Of the five ER clones derived from MCF10AneoT cell cDNA, one clone exhibited a base substitution at codon 286 in the hinge region of the ER molecule (ATG→ACG) which will lead to replacement of methionine by threonine. The presence of

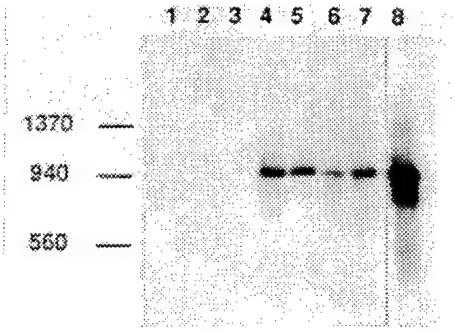

Figure 1. Southern blot analysis of RT-PCR amplified ER cDNAs in the MCF10AT system. Lane 1 contains ER DNA amplified from MCF10A; lane 2, MCF10Aneo; lane 3, MCF10AneoN; lane 4, MCF10AneoT; lane 5, MCF10AT1; lane 6, MCF10AT2b, and lane 7, MCF10AT3c. Lane 8, denotes ER DNA amplified from a plasmid containing the full length human ER DNA sequence. Amplified DNA fragments were subjected to Southern blot hybridization analysis with a full length human ER DNA probe. Note that the 967 bp fragment amplified by RT-PCR using ER specific primers is detectable only in cDNAs amplified from MCF10AT and other cells of the MCF10AT system.

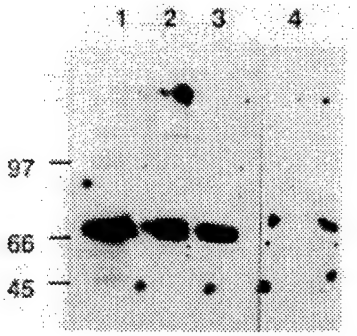

Figure 3. Detection of ER protein by immunoprecipitation and Western blot analysis in MCF-7 and cells of MCF10A derivatives. 100 µg (MCF-7) to 200 µg (MCF10AneoT, MCF10AT3c, MCF10Aneo) of cytosolic protein was used for each immunoprecipitation with 10 µg/ml of anti-ER monoclonal antibody D547. For Western blot analysis, 3 µg/ml of the same antibody was used. Lane 1, represents MCF-7; lane 2, MCF10AT3c; lane 3, MCF10AneoT and lane 4, MCF10Aneo. Note the absence of immunoreactivity with ER monoclonal antibody in MCF10Aneo cells.

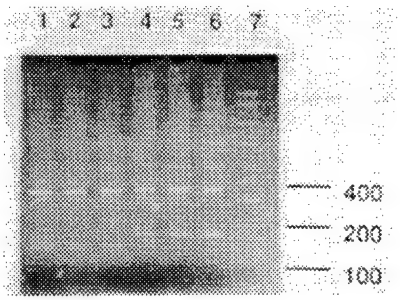

Figure 2. Detection of mutated (T24) *c-Ha-ras* mRNA expression in cells of the MCF10AT system. *Nae*I-restriced *Ha-ras* cDNAs from: lane 1, MCF10A; lane 2, MCF10Aneo; lane 3, MCF10AneoN; lane 4, MCF10AneoT; lane 5, MCF10AT1; lane 6, MCF10AT3c; lane 7, molecular size markers. Note the novel 179 bp bands in lanes 4-6, indicating the presence of cDNAs with a single base RAS activating substitution at codon 12. See Materials and methods for details.

this sequence alteration has been confirmed by a second independent PCR reaction indicating that the identified mutation is not an artifact of PCR reaction. All four ER clones derived from MCF10AT3c and the remaining four ER clones derived from MCF10AneoT cells had sequences identical to the sequence for normal human ER reported in GenBank (25). These results suggest that expression of the bacterial neomycin resistance gene in MCF10Aneo and the normal *c-Ha-ras* gene in MCF10AneoN cells are not capable of activating the ER gene in MCF10A cells, but that expression of mutated (T24) *c-Ha-ras* may play a role. Steady-state levels of ER transcription in MCF10AT system cells are low since we have not been successful in demonstrating ER expression by Northern blot analysis, even when loading was increased to 10 µg of poly A^+ RNA.

Localization and analysis of expression of T24 c-Ha-ras in the MCF10AT system. Previous studies (26) indicated that the T24 *c-Ha-ras* gene(s) transfected into MCF10AT cells were present at a single insertion site. FISH analysis of chromosomes was utilized to localize this site and rule out the formal possibility that the ER gene activation was the result of nearby insertion of pHO6T1, a plasmid carrying transcriptional enhancers from the Moloney murine sarcoma virus and the simian virus 40 along with the T24 *c-Ha-ras* gene. FISH analysis of MCF10AT and MCF10AT3c cells detected a single site homologous to the pHO6T1 probe (data not shown). This site was not present in MCF10A cells. Karyotypic analysis indicated that pH06T1 is inserted on the q arm of one A group chromosome (1, 2 or 3) in MCF10AT cells. Thus, it cannot cause direct positional activation of ER which is on chromosome 6q (27).

RT-PCR with *c-Ha-ras*-specific primers was used to determine if the transfected T24 *Ha-ras* oncogene present in MCF10AneoT cells was expressed as mRNA in other lines of the MCF10AT system. Amplified PCR products were subjected to *Nae*I digestion to distinguish between cDNA fragments derived from transcripts of the T24 *Ha-ras* gene in the vector pHO6T1 and those derived from endogenous genes. The 179 bp T24 *Ha-ras*-specific fragment diagnostic for the activating GCC→GTC substitution in codon 12 was only present in PCR amplified cDNA from MCF10AT and its derivatives (Fig. 2, lanes 4-6). This fragment was readily distinguishable from the 144 bp *Nae*I fragment of normal *Ha-ras* cDNA which was present in amplified cDNA from MCF10A, MCF10AneoT and their derivatives (Fig. 2, lanes 1-3, and lower bands in lanes 4-6). Although it does not prove a causal relationship, this result demonstrates that the only MCF10A derived lines that express ER are those that also express T24 *Ha-ras*.

Detection of ER protein. Sequence analysis indicated that MCF10AT cells should be capable of synthesizing normal ER. To confirm that ER protein was present in MCF10AT system cells, proteins obtained by immunoprecipitation with anti-human ER rat monoclonal antibody D547 were analyzed. MCF10AT3c cells were selected as a representative 'progressed

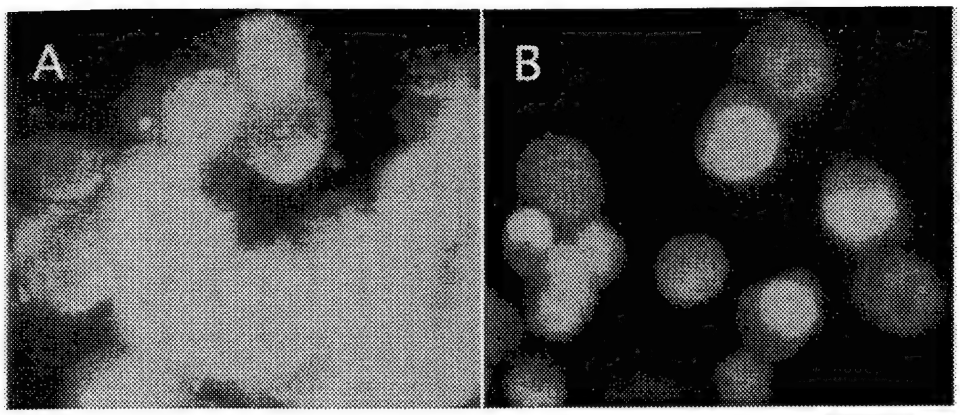

Figure 4. Immunocytochemical detection of ER in MCF-7 (Panel A) and MCF10AT3c cells (Panel B). Cells were incubated with anti-human ER monoclonal antibody H222 followed by fluorescein tagged rabbit anti-rat IgG following recommended procedures of the supplier (Abbot Laboratories). Note that under identical conditions of preparation, intensity of nuclear fluorescence in MCF-7 cells is stronger than in MCF10AT3c cells and that, at similar cell density, a greater percentage of MCF-7 cells have high levels of nuclear protein capable of binding the anti-ER antibody. Controls, cells incubated either without mAb-H222 or with non-immune rat IgG, lacked any nuclear fluorescence (data not shown).

line', and because a greater proportion of these cells express detectable ER protein. Lysates from both MCF10AT3c and MCF10AneoT cells (Fig. 3, lanes 2 and 3) contained a single molecular size species of ER protein. This ~67 kDa protein had the same electrophoretic mobility as ER from MCF-7 cells (Fig. 3, lane 1). Quantitation by densitometry indicated that the levels of ER protein were at least 5 to 10-fold lower in MCF10AT3c and MCF10AneoT cells, respectively, than in the positive control, MCF-7 cells. As expected, no ER protein was detected in lysates from MCF10Aneo cells (Fig. 3, lane 4). Although ER was detected in MCF10AneoT cells by Western blotting (Fig. 3) and flow cytometry (data not shown), we have not been able to detect it reproducibly by immunocytochemical staining with anti-human ER rat monoclonal antibody H222 even though the same antibody can detect ER in MCF10AT3c cells (Fig. 4). The intensity of fluorescent staining was lower in MCF10AT3c nuclei than in MCF-7 nuclei. However, under optimal conditions of active growth to near confluence, nuclear ER was detectable in approximately 30-40% of MCF10AT3c cells compared to >80% of MCF-7 cells (Fig. 4).

Functional status of endogenous ER in MCF10AT cells. Although our results demonstrated that cells of the MCF10AT system were capable of synthesizing ER that localized to the nucleus, it was still necessary to establish that this ER was functionally active, i.e., that it could mediate transcriptional activation. This was assessed by measuring the effect of E_2 on transient expression of the bacterial CAT reporter gene in JA-12, a plasmid with CAT expression regulated by tandem EREs upstream of a minimal TK promoter (17) and by determining the effect of E_2 on expression of the endogenous PgR genes in MCF10AT cells.

CAT expression. A typical assay of CAT activities obtained after transfection of MCF10AneoT cells with JA-12 is illustrated, and results of three independent transfections are summarized in Fig. 5. Extracts from MCF10AneoT cells transfected with JA-12 and grown in the absence of E_2 had approximately 2-fold higher levels of CAT activity than those transfected with control, enhancerless minimal promoter construct p-37TKCAT (18). Induction of CAT expression was observed in MCF10AneoT cells cultured in the presence of as little as 10^{-10} M E_2. At this concentration, the level of CAT in MCF10AneoT lysates was able to acetylate ~4% of input chloramphenicol in 4 h. Maximal induction of CAT expression (~32% conversion of input chloramphenicol in 4 h) was obtained with 10^{-8} M E_2. The level of CAT activity in lysates from MCF10AneoT cells transfected with p-37TKCAT was unaffected by exposure of the cells to E_2 (data not shown). The E_2 antagonist, ICI 164,384, was used to establish the specificity of E_2-induced response in MCF10AneoT cells. ICI 164,384 at a 100-fold higher concentration than E_2 (10^{-7} vs 10^{-9} M), reduced E_2-induced CAT activity by 50% (Fig. 5). Co-transfection with pCH110, which expresses ß-galactosidase activity at a constant level regardless of E_2 concentration, confirmed that these results were not due to differences in transfection efficiency or cell viability (data not shown). The functional status of ER could not be reliably determined by transient reporter assay in MCF10AT3c cells because of the low level of CAT expression obtained with calcium-mediated transfection. This is most likely due to poor uptake of DNA, since low levels of CAT and ß-galactosidase expression were also obtained after transfection of these cells with pCAT and pCH110, respectively.

PgR or PgR-related protein expression. Levels of PgR protein were determined in cell lysates from MCF10AneoT, MCF10AT3c, two E_2-responsive MCF10AT3c clones (selected from soft agar growth assays) and PgR-positive breast cancer cell line, T47D, by immunoprecipitation and Western blotting analysis. The majority of PgR protein detected by PgR monoclonal antibody, Ab-1 (Oncogene Science, Cambridge, MA), from lysates of T47D cells had an electrophoretic mobility corresponding to that of the A (Mr ~83 kDa) and B (Mr ~110 kDa) forms of PgR with a trace of protein with an apparent Mr ~50 kDa (Fig. 6A, lane 1). In contrast, the bulk

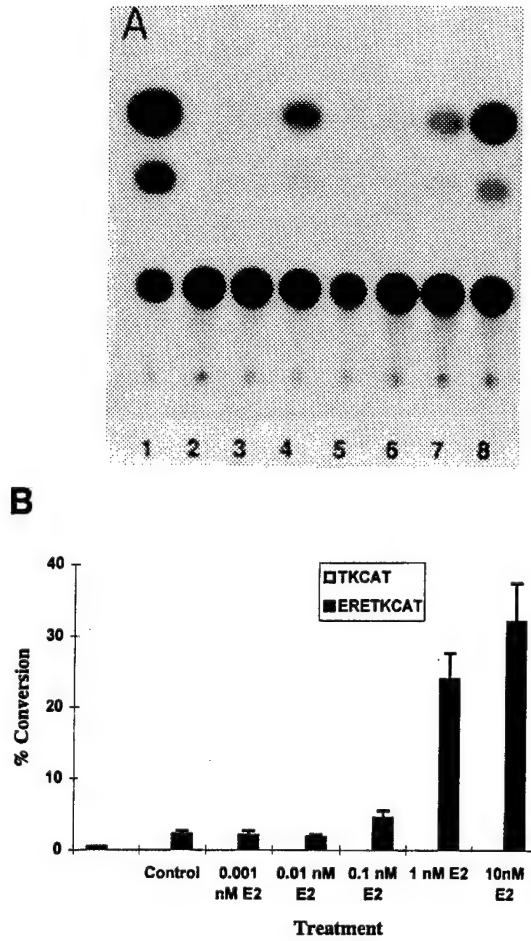

Figure 5. Functionality of ER by CAT expression from a transiently transfected ERE-TK-CAT gene in MCF10AneoT cells. Panel A, a typical autoradiogram showing CAT activity in MCF10AneoT cells transfected with the plasmid JA-12 and treated for ~40 h with E_2 over a concentration range of 10^{-12}-10^{-9} M. Lane 1, cells transfected with the hormone-independent plasmid, pCAT containing SV40 promoter and enhancer sequences with the CAT gene; lane 2, p(-37)TK-CAT; lanes 3-8 represent cells transfected with the plasmid JA-12: lane 3, control (minus E_2); lane 4, 10^{-9} M E_2 plus 10^{-7} ICI 164,384; lane 5, 10^{-12} M E_2; lane 6, 10^{-11} M E_2; lane 7, 10^{-10} M E_2 and lane 8 represents cells treated with 10^{-9} M E^2. Panel B, results obtained from three independent transfections are summarized and graphically represented. Maximal conversion of chloramphenicol was observed in presence of 10^{-8} M E_2 (not shown in panel A). Co-transfection with pCH110 which expresses ß-galactosidase activity at a constant level regardless of E_2 concentration confirmed that the results were not due to differences in transfection efficiency or cell viability (data not shown).

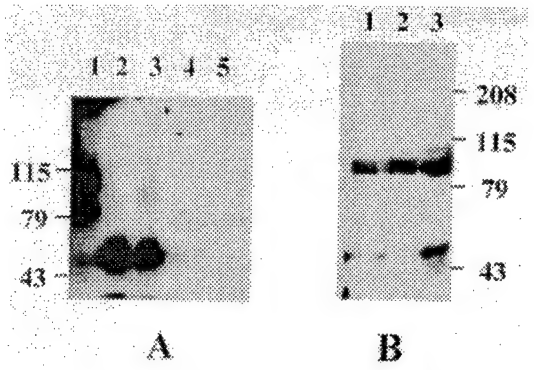

Figure 6. Detection of PgR or PgR-related proteins in MCF10AT system cells by immunoprecipitation and Western blot analysis. Anti-PgR monoclonal antibody at a concentration of 10 µg/ml was used for immunoprecipitation of PgR from cytosols of E_2-treated and untreated MCF10AneoT and MCF10AT3c cells, E_2-treated MCF10AT3c clones, and T47D cells, a constitutive expressor of PgR. For Western blot analysis of immunoprecipitated samples, 5 µg/ml of the same PgR antibody was used. A, lane 1 represents the positive control, T47D cells; lanes 2 and 3 represent MCF10AneoT and MCF10AT3c cells treated with 10^{-9} M E_2, respectively; lanes 4 and 5 represent MCF10AneoT and MCF10AT3c cells without treatment with E_2. B, lanes 1 and 2 represent MCF10AT3c clones A and B treated with 10^{-9} M E_2, respectively; lane 3 represents T47D cells.

of the protein that bound anti-PgR Ab-1 antibody from lysates of E_2-treated MCF10AneoT and MCF10AT3c cells had the mobility of a 50 kDa protein and contained only trace amounts of PgR protein with the mobility of A form PgR and no detectable B form (Fig. 6A, lanes 2 and 3). The PgR-A protein in MCF10AT3c cells migrated slightly slower than those seen in the positive control breast cancer cell line T47D and MCF10AneoT cells. If the observed differences in mobility are not the result of differences in protein loading, then it is possible that the PgR A forms in the tested cell lines differ in level or number of phosphorylation sites, primary structure or other post-translational modifications (28). Samples prepared from untreated MCF10AneoT and MCF10AT3c cells had barely detectable levels of the 50 kDa protein and none of the larger forms (Fig. 6A, lanes 4 and 5). Interestingly, similar analysis of PgR proteins from lysates of two E_2-responsive clones of MCF10AT3c cells (selected and cloned on the basis of estrogen-enhanced growth in soft agar and E_2-induced effects on cell cycle progression) revealed majority of PgR immunoreactive protein to correspond with A (Mr ~83 kDa) form of PgR with no detectable B form, and only trace amounts of the 50 kDa protein (Fig. 6B, lanes 1 and 2). As the PgR epitope recognized by the monoclonal antibody Ab-1, used in these experiments has not been localized, the origin of the E_2 inducible 50 kDa protein observed in MCF10AneoT and MCF10AT3c cell populations remains to be established.

Effect of E_2 on anchorage-independent growth of MCF10AT3c cells. Monolayer growth of MCF10A cells and MCF10A cells rendered ER-positive after stable transfection with an ER expression plasmid is unaffected or decreased, respectively, by addition of E_2 to the growth medium (10). We have found no significant effect of E_2 on monolayer proliferation of MCF10AneoT cells (data not shown). MCF10A cells do not form colonies in soft agar (9). However, both transfection with T24 *Ha-ras* (7) and transplant passage result in enhanced capacity for anchorage-independent growth. Under the conditions of assay utilized in our experiments, MCF10AneoT cells had a colony forming efficiency (CFE) of 10% with the majority of the colonies smaller than 100 µm in diameter. E_2 had no significant effect on either the CFE or the average colony size of MCF10AneoT cells (data not shown). However, E_2 treatment did lead to the rare appearance of colonies with

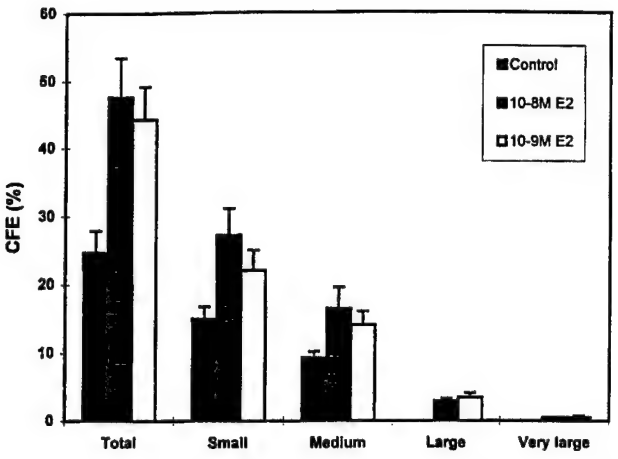

Figure 7. Effect of E_2 on anchorage-independent growth of MCF10AT3c cells. Total number of colonies represent colonies greater than 50 μm in diameter. Colonies were counted according to their size: small, medium, large, and very large, 50-100 μm, 100-200 μm, 200-300 μm and >300 μm in diameter, respectively. Ten microscopic fields were counted to calculate the number of colonies for the whole well, and average of 3 wells for treated (10^{-9} and 10^{-8} M E_2) or untreated cells was calculated. Results are expressed mean ± SD of 3 experiments.

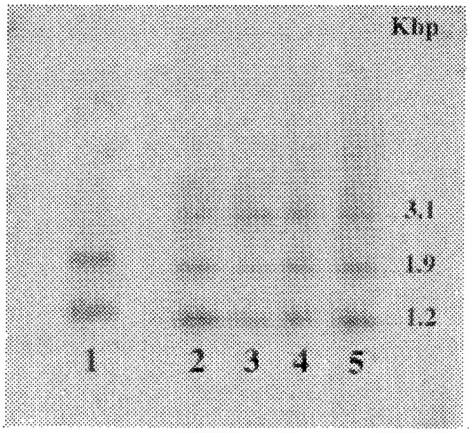

Figure 8. The methylation sensitive restriction enzyme *Not*I was used to test the methylation status of a representative site in the exon1 CpG island of the ER gene. After *Eco*RI, *Not*I cleavage, a 3.1 kbp fragment is detected by a probe specific for this region (See Materials and methods) if the *Not*I site is methylated. If the site is unmethylated, 1.9 and 1.2 kbp fragments result. DNA from MCF-7 cells (lane 1); MCF10A cells (lane 2); MCF10Aneo cells (lane 3); MCF10AneoT cells (lane 4) and MCF10AT3c cells (lane 5). Note that the proportion of unmethylated (1.9 kbp) *Eco*RI fragment is identical in lane 2 (ER-negative parental MCF10A cells) and lanes 4, 5 (ER-positive MCF10AT system cells).

diameters >200 μm (~1/8x10^4 colonies). In the absence of E_2, MCF10AT3c cells had a CFE of 25% and approximately 40% of these colonies had a diameter >100 μm; none had a diameter >200 μm. The CFE of MCF10AT3c cells showed a dramatic increase when the cells were exposed to physiological concentrations of E_2 (Fig. 7). E_2 treatment also enhanced the rate of growth in soft agar with ~1/40-80 colonies larger than 200 μm.

Correlation of ER expression with the methylation status of ER gene in the MCF10AT system. The ER gene contains several CpG islands in its promoter and first exon that are rich in sites recognized by methylation sensitive restriction endonucleases (29). There are several reports indicating that methylation of a number of these sites is correlated with silencing of ER (30,31). The methylation status of the CpG island in exon 1 was examined by Southern blot analysis of DNA digested with the methylation sensitive enzyme, *Not*I, and the methylation insensitive enzyme, *Eco*RI. The 3.1 kbp *Eco*RI fragment containing this CpG island has a single *Not*I site. *Not*I cleaves the 3.1 kbp fragment, releasing 1.2 and 1.9 kbp fragments if the site is completely unmethylated (31). Our results (Fig. 8) indicate that this *Not*I site is unmethylated in most MCF10A cells and that the proportion of methylated to unmethylated *Not*I sites in exon1 does not change in MCF10AneoT and its derivatives. This suggests that even though loss of methylation at this *Not*I site may be an indicator of a generalized lack of methylation in the CpG islands of the ER and of active ER transcription in breast tumor cells and lines (30,31), loss of methylation at the *Not*I site in exon 1 is not sufficient to allow activation of ER transcription in MCF10A cells.

Discussion

By examining gene expression in a number of the cell lines that comprise the MCF10AT xenograft model for progression of human proliferative breast disease, we have demonstrated that spontaneous expression of endogenous ER occurred only in MCF10A-derived lines that are transfected with and expressing T24 *Ha-ras*. Although the steady-state levels of ER transcripts are very low, our data show that the ER protein expressed in cell lines of the MCF10AT system is detected by immunoprecipitation/Western blot analysis suggesting efficient translation of ER mRNAs in MCF10AT system cells. Our data further indicate that the ER expressed in cell lines of the MCF10AT system is functional and can mediate E_2 induced transcription of both endogenous and transfected genes as well as stimulation of colony formation and anchorage-independent growth of MCF10AT3c cells in soft agar.

Our studies revealed that the majority of estrogen-induced PgR (or PgR-related protein) in MCF10AneoT and MCF10AT3c cell populations has a molecular size of ~50 kDa rather than the 83 and 110 kDa A and B forms observed in T47D breast cancer cells (Fig. 6A; ref. 32) and normal human tissues (33). However, E_2-responsive clones of MCF10AT3c cells that were selected on the basis of estrogen-induced growth in anchorage-independent growth assays and cell cycle kinetics differed from the parental MCF10AT3c cells in that they expressed the 83 kDa A form of PgR. This would suggest that only a small proportion of MCF10AT3c population expresses PgR A whereas majority of MCF10AneoT and MCF10AT3c cells express the smaller 50 kDa PgR or PgR-related protein. Translation from an internal initiation codon AUG encoded in exon 2 (Met^{595}) has been shown to lead to synthesis of a 45-50 kDa PgR termed the C receptor (28). Preliminary examination of estrogen-induced transcripts by RT-PCR using PgR-specific primers and Southern blot hybridization analysis suggest that the 50 kDa protein observed in cellular extracts

of estrogen-treated MCF10AT cells may represent a PgR variant that is distinct from the C form or a PgR-related protein (P.V.M. Shekhar and F. Saifer, unpublished data). Ongoing molecular and functional characterization of the 50 kDa protein should determine a) the relationship between the 50 kDa protein and the 83 kDa PgR or b) whether the 50 kDa protein is a PgR-like protein. Besides these effects of estrogen on expression of PgR or PgR-like proteins in MCF10AT cells, we have also recently demonstrated ER functionality in MCF10AT3c cells at the level of induction of pS2 mRNA, a known estrogen-regulated target gene (34). Our results have not provided many clues as to the mechanism(s) by which ER transcription became activated after transfection of MCF10A cells with T24 *Ha-ras*. However, results from karyotypic analysis has indicated that spontaneous acquisition of ER gene function in MCF10AT cells is not the result of direct positional activation due to nearby insertion of the T24 *Ha-ras* encoded plasmid. Although not all studies are in agreement (35,36), there are a number of reports indicating that the silencing of ER expression in breast tumors and breast tumor cell lines is associated with extensive methylation of C residues in the exon 1 CpG island of ER (30,31,37), including those in the *Not*I site examined in the studies reported here. In ER-negative MDA-MB-431 breast cancer cells, this site is completely methylated as indicated by resistance to *Not*I cleavage (31,38). Treatment with 5-azadeoxycytidine, which is incorporated into DNA where it acts as an inhibitor of 5-C-DNA methyltransferase (39,40), results in partial loss of methylation at a number of CpG sites in these cells, including the exon 1 *Not*I site (38). It also results in expression of functional ER protein, strengthening the suggestion that methylation plays a role in silencing ER transcription (39). Our data demonstrate i) that lack of methylation at the *Not*I restriction site cannot by itself be used as a direct indicator of transcriptional activation of the ER gene in breast cells and ii) that a low level of methylation at the *Not*I site, which is characteristic of normal breast tissue (30), is not sufficient to allow expression of ER in MCF10A cells. The low level of methylation at this *Not*I site is similar in both ER-negative MCF10A cells and ER-positive MCF10AneoT and MCF10AT3c cells (Fig. 8). A similar lack of correlation between the methylation status of a CpG island and ER expression has been recently reported by Chen *et al* (41) who demonstrated that methylation of CpG island is not required for the loss of ER expression in ER-negative breast cancer cells that are derived from ER-positive cells. However, these results do not rule out the possibility that methylation of other C residues at upstream sites in the ER promoter may play a role in regulating ER expression. We are currently evaluating this by analyzing methylation of all C residues in the promoter and exon 1 regions of MCF10A and MCF10AneoT ER genes.

An alternative mechanism for transcriptional activation of the ER gene that should be considered is that expression of a mutated *Ha-ras* gene leads to alterations in levels or activity of transcription factors that enhance ER transcription. Since *Ha-ras* mutations are rare in human breast cancer, there has been speculation that T24 *Ha-ras* expression mimics the effect of some more common, as yet unidentified, genetic defect that leads to development of breast cancer (6,8). However, since overexpression of normal *Ha-ras* is a property of both benign proliferative and malignant human breast tissues (42), it is possible that low level expression of mutated *Ha-ras* is equivalent to a high level expression of normal *Ha-ras* in maintaining growth potential of human breast epithelial cells *in vivo*. As oncogenic Ras proteins are insensitive to GAP stimulation, they consequently persist chronically in the GTP-bound state, resulting in the constitutive activation of downstream growth regulatory pathways (42). Our data indicate that a certain threshold level of *Ha-ras* may be required for promoting neoplastic progression, and that MCF10AneoN cells probably fail to produce persistent lesions because the level of *Ha-ras* expression in these cells is not significantly higher than in untransfected MCF10A cells (Fig. 2 and unpublished results).

Our studies show that expression of ER in MCF10AneoT cells is not sufficient to allow an E_2 induced increase in either the potential for anchorage-independent growth (CFE) or the average rate of growth of colonies. This is consistent with the finding that the sex of the host had little or no influence on the establishment or growth of MCF10AneoT lesions (F. Miller, unpublished data). However, E_2 treatment *in vitro* did allow detection of a small population of MCF10AneoT cells with the potential to form large (>200 μm) colonies in soft agar. In contrast, MCF10AT3c cells, which had undergone three rounds of selection for ability to form lesions *in vivo*, responded to E_2 treatment with a significant increase in both CFE and colony size. This result, combined with the preliminary observation that MCF10AT1 cells formed lesions with elements of invasive carcinoma only in female mice (F. Miller, unpublished data), suggests the possibility that while E_2/ER-mediated processes are not necessary for growth and/or establishment of lesions by MCF10AneoT cells, they greatly enhance the growth potential of a small subset of ER-positive cells in the MCF10AneoT population and that this population is enriched in MCF10AT lesions by xenograft passage. This leads to the predictions that i) E_2-responsive ER-positive cells will be enriched relative to ER-negative cells in the most progressed areas of MCF10AT system lesions and ii) an increase in degree of progression and/or size of the most progressed areas of lesion will occur in hosts with elevated E_2 levels relative to those lacking E_2. Studies of the histology and expression of selected genes in lesions from E_2 supplemented hosts as compared to anti-estrogen treated hosts are currently underway to test these hypotheses.

In summary, although the underlying mechanisms of ER activation in MCF10AneoT cells and the role of RAS in development of human breast tumors remain unclear, the studies reported here indicate that the presence of a functionally active endogenous ER in MCF10AT xenograft model provides us with a unique opportunity to understand molecular alterations in early breast cancer and the role of estrogenic compounds in neoplastic progression of human breast epithelial cells.

Acknowledgements

The authors thank Dr Geoffrey Greene for generously supplying the anti-ER monoclonal antibody D547. The authors also thank the late Dr H.D. Soule for his helpful

suggestions regarding soft agar assays, and Drs Avraham Raz and Sandra Wolman for critical reading of the maunuscript. The research was supported by grants from the U.S. Army Medical Research and Materiel Command (DAMD17-94-J-4427; PVMS, JKC), National Institutes of Health (CA60881, PVMS), CA22453 (Core grant to the Karmanos Cancer Institute), Elsa U. Pardee Foundation (GHH), and the Nebraska Cancer and Smoking Disease Research Program (JKC).

References

1. Petersen OW, Hoyer PE and van Deurs B: Frequency and distribution of estrogen receptor-positive cells in normal, non-lactating human breast tissue. Cancer Res 47: 5748-5751, 1987.
2. Jacquemier JD, Hassoun J, Torrente M and Martin PM: Distribution of estrogen and progesterone receptors in healthy tissue adjacent to breast lesions at various stages - immunohistochemical study of 107 cases. Br Cancer Res Treat 15: 109-117, 1990.
3. McGuire WL, Carbone PP, Sears ME and Eschert GC: Estrogen receptors in human breast cancer: an overview. In: Estrogen Receptors in Breast Cancer. McGuire WL, Carbone PP and Voller EP (eds). Raven Press, New York, pp1-7, 1975.
4. Lippman ME and Dickson RB: In: Breast Cancer: Cellular and Molecular Biology. Kluwer Academic Publishers, Boston, pp119-165, 1988.
5. Wittligg JL: Steroid hormone receptors in breast cancer. Cancer 53: 630-643, 1984.
6. Miller FR, Soule HD, Tait L, Pauley RJ, Wolman SR, Dawson PJ and Heppner GH: Xenograft model of human proliferative breast disease. J Natl Cancer Inst 85: 1725-1732, 1993.
7. Basolo F, Elliot J, Tait L, Chen XQ, Maloney T, Russo IH, Pauley R, Momiki S, Caamano J, Klein-Szanto AJ and Russo J: Transformation of human breast epithelial cells by c-*Ha-ras* oncogene. Mol Carcinogen 4: 25-35, 1991.
8. Dawson PJ, Wolman SR, Tait L, Heppner GH and Miller FR: MCF10AT: a model for the evolution of cancer from proliferative breast disease. Am J Pathol 148: 313-319, 1996.
9. Soule HD, Maloney TM, Wolman SR, Peterson WD, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF and Brooks SC: Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 50: 6075-6086, 1990.
10. Pilat MJ, Christman JK and Brooks SC: Characaterization of the estrogen receptor transfected MCF10A breast cell line 139B6. Breast Cancer ResTreat 37: 253-266, 1996.
11. Wiese TE, Kral LG, Dennis KE, Butler WB and Brooks SC: Optimization of estrogen growth response in MCF-7 cells. In Vitro Cell Dev Biol 28A: 595-602, 1992.
12. Green GL, Gilna P, Waterfield M, Baker A, Hert Y and Shine J: Sequence and expression of human estrogen receptor complementary DNA. Science 231: 1150-1154, 1986.
13. Reddy EP, Reynolds RK, Santos E and Barbacid M: A point mutation is responsible for the acquisition of transforming properties by the T24 bladder carcinoma oncogene. Nature 300: 149-152, 1982.
14. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
15. Talmadge CB, Zhen D, Wang JY, Berglund P, Li BF, Weston MD, Kimberling WJ, Zabarovsky ER, Stanbridge EJ and Klein G: Construction and characterization of a NotI linking library from human chromosome region 1q25-qter. Genomics 29: 105-114, 1995.
16. Spandidos DA and Wilkie NM: Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature 310: 469-475, 1984.
17. Vander-Kuur JA, Hafner MS, Christman JK and Brooks SC: Effects of estradiol-17ß analogues on activation of estrogen response element regulated chloramphenicol acetyltransferase expression. Biochemistry 32: 7016-7021, 1993.
18. Strahle U, Schmid W and Shutz G: Synergistic action of the glucocorticoid receptor with transcriptional factors. EMBO J 7: 3389-3395, 1988.
19. Graham F and van der Eb A: A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52: 456-467, 1973.
20. Gorman CM, Moffat LF and Howard BH: Recombinant genome which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2: 1044-1051, 1982.
21. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976.
22. Current Protocols in Molecular Biology. Chapter 2. Unit 2.2. John Wiley & Sons, Inc.
23. Nelson M and McClelland M: Effect of site-specific methylation on DNA modification methyltransferases and restriction endonucleases. Nucleic Acids Res 17 (Suppl.): 389-415, 1989.
24. Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE and Baylin SB: Methylation of the oestrogen receptor CpG island links ageing and neoplasia. Nat Genet 7: 536-540, 1994.
25. Locus HUMERMCF, accession M12674.
26. Miller FR, Pauley RJ and Wang B: Activated c-Ha-*ras* is not sufficient to produce the preneoplastic phenotype of human breast cell line MCF10AT. Anticancer Res 16: 1765-1769, 1996.
27. Schuler GD, Boguski MS, Stewart EA, Stein LD, Gyapay G, Rice K, White RE, Rodriguez Tome P, Aggarwal A, Bajorek E, Bentolila S, Birren BB, Butler A, Castle AB, Chiannilkulcha, N, Chu A, Clee C, Cowles S, Day PJ, Dibling T, Drouot N, Dunham T, Duprat S, East C, Hudson TJ, *et al:* A gene map of the human genome. Science 274: 540-546, 1996.
28. Wei LL, Gonzalez-Aller C, Wood WM, Miller LA and Horowitz KB: 5' heterogeneity in human progesterone receptor transcripts predicts a new amino terminal truncated 'C'-receptor and unique A-receptor messages. Mol Endocrinol 4: 1833-1840, 1990.
29. Locus HSERB5FR, accession X62462.
30. Lapidus RG, Ferguson AT, Ottaviano YL, Parl FF, Smith HS, Weitzman SA, Baylin SB, Issa J-P and Davidson NE: Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clin Cancer Res 2: 805-810, 1996.
31. Ottaviano YL, Issa J-P, Parl FF, Smith HS, Baylin SB and Davidson NE: Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res 54: 2552-2555, 1994.
32. Lessey BA, Alexander PS and Horowitz KB: The subunit structure of human breast cancer progesterone receptors: characterization by chromatography and photoaffinity labeling. Endocrinology 112: 1267-1274, 1983.
33. Feil PD, Clarke CL and Satyaswaroop PG: Progestin mediated changes in progesterone receptor forms in the normal human endometrium. Endocrinology 123: 2506-2513, 1988.
34. Shekhar PVM, Werdell J and Basrur VS: Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. J Natl Cancer Inst 89: 1774-1782, 1997.
35. Falette NS, Fuqua SA, Chamness GC, Cheah MS, Greene GL and McGuire WL: Estrogen receptor gene methylation in human breast tumors. Cancer Res 50: 3974-3978, 1990.
36. Kass DH, Shen M, Appel NB, Anderson DE and Saunders GF: Examination of DNA methylation of chromosomal hot spots associated with breast cancer. Anticancer Res 13: 1245-1251, 1993.
37. Piva R, Rimondi AP, Hanau S, Maestri I, Alvisi A, Kumar VL and del Senno L: Different methylation of oestrogen receptor DNA in human breast carcinomas with and without oestrogen receptor. Br J Cancer 61: 270-275, 1990.
38. Ferguson AT, Lapidus RG, Baylin SB and Davidson NE: Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res 55: 2279-2283, 1995.
39. Creusot F, Acs G and Christman JK: Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'-deoxycytidine. J Biol Chem 257: 2041-2048, 1982.
40. Taylor SM and Jones PA: Mechanism of action of eukaryotic DNA methyltransferase: use of 5-azacytosine containing DNA. J Mol Biol 162: 679-692, 1982.
41. Chen Z, Ko A, Yang J and Jordan VC: Methylation of CpG island is not a ubiquitous mechanism for the loss of estrogen receptor in breast cancer cells. Br J Cancer 77: 181-185, 1998.
42. Clark GJ and Der CJ: Aberrant function of the ras signal transduction pathway in human breast cancer. Breast Cancer Res Treat 35: 33-144, 1995.

ARTICLES

Environmental Estrogen Stimulation of Growth and Estrogen Receptor Function in Preneoplastic and Cancerous Human Breast Cell Lines

*P. V. M. Shekhar, J. Werdell, V. S. Basrur**

Background: **DDT and polychlorinated biphenyls (PCBs), which are widespread in the ecosystem, can mimic estrogen-mediated cell activities. Thus, they can potentially interfere with many physiologic processes. We compared the effects of organochlorines belonging to the DDT and PCB families, alone and in combination, for their ability to influence the estrogen receptor-mediated activities in preneoplastic breast epithelial cells and breast cancer cells.** ***Methods:*** **Multiple assay systems requiring functional estrogen receptor were employed to test estrogen-like activity of organochlorine ligands. Two-sided statistical tests were used to compare the data.** ***Results:*** ***p,p′*****-DDT, the predominant form of DDT in the environment, is a more potent estrogen than** ***o,p′*****-DDT (*P*<.001), although it is less effective than** ***o,p′*****-DDT in inhibiting the binding of estradiol (natural estrogen) to estrogen receptor. Among the PCBs, Heptachlor is estrogenic (in transient reporter assays; *P*≤.001), whereas Aroclor 1221 and Aroclor 1254, both individually and in combination, are only weakly estrogenic.** ***Conclusion:*** ***p,p′*****-DDT is the most effective organochlorine in regulating estrogen receptor-mediated cellular responses. In estrogen receptor-positive breast cancer cells,** ***p,p′*****-DDT evokes responses by itself and enhances the responses in collaboration with estradiol or** ***o,p′*****-DDT. [J Natl Cancer Inst 1997;89:1774–82]**

Epidemiologic studies imply that hormones, genetic factors, and environmental agents are important determinants in breast carcinogenesis. A wide variety of pollutants in the ecosystem have the ability to mimic the actions of steroid hormones in the body. Principal concern is centered around chemicals that can mimic the actions of estrogens, termed "environmental estrogens" or "xenoestrogens," since they have the ability to interfere with several physiologic processes that are normally estrogen regulated, which could result in alterations in reproduction and susceptibility to cancer *(1–10)*. Although there is great concern over the role of xenoestrogens in the development of breast cancer, there are little data to support their role. Estrogen hormones are potent mitogens for breast epithelial and uterine cells, whose hormone responsiveness is mediated by the estrogen receptor (ER). The mechanism by which estrogens affect proliferation of certain target cells is not fully understood *(11,12)*. Several lines of evidence indicate that they can induce expression of cell cycle regulatory genes directly *(13–18)*, suggesting a primary role for ER in regulating cell cycle progression.

Organochlorines are lipophilic compounds that accumulate and persist in human adipose tissue, reaching levels 200–300 times higher than those observed in serum *(19–21)*. The following are well-known examples of organochlorines: the pesticide DDT, i.e., 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane, and its metabolite DDE, i.e., 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene *(19–24)*, and PCBs, i.e., polychlorinated biphenyls *(19–23,25)*. To date, DDT has been shown to induce cancers of the liver, lymphatic tissue, and lung in rats and mice *(20,21,26,27)* but not mammary cancer. At a molecular level, DDT can induce cytochrome P450-containing enzymes in humans, thus potentially affecting steroid metabolism *(20,21,26,27)*. *p,p′*-DDT, the predominant form of DDT, has been considered nonestrogenic *(28)*, whereas *o,p′*-DDT and its isomer *o,p′*-DDE are estrogenic *(24,29,30)*. *o,p′*-DDT has been shown to promote growth of rat mammary tumors *(31,32)* and to bind to the ER of rat and human mammary tumors *(33,34)* and human uterine tumors *(34)*. Similarly, Aroclor 1221, a mixture of PCBs that is rich in *o*-chlorobiphenyl, has been reported to be estrogenic, whereas Aroclor 1254 is nonestrogenic *(35)*.

In this study, we have compared the effects of closely related organochlorines belonging to the DDT (*p,p′*-DDT and *o,p′*-DDT) and PCB (Aroclor 1221, Aroclor 1254, and Heptachlor) families singly and in mixtures for estrogenicity and ER-mediated properties in two cell systems of human breast cancer: ER-positive preneoplastic breast epithelial cells (MCF10AT cells) and ER-positive breast carcinoma (MCF-7 cells).

Materials and Methods

Preneoplastic Breast Epithelial Cell Model System

The MCF10AT system is a xenograft model of early human breast cancer progression *(36)*. MCF10AneoT cells are T24 Ha-ras-transformed cells derived

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See "Notes" following "References."

from MCF10A human breast epithelial cells *(37)*. MCF10A cells do not form persistent lesions in immunodeficient mice *(36)*, whereas MCF10AneoT cells do *(36)*. MCF10AneoT and lines derived by alternating *in vivo* transplantation and *in vitro* culture (MCF10ATn) are collectively known as the MCF10AT system *(38)*. The lesions formed by lines of the MCF10AT system are composed of a heterogeneous spectrum of ductular tissues with a range of morphology that includes mild hyperplasia, atypical hyperplasia, carcinoma *in situ* (CIS), moderately differentiated carcinoma, and undifferentiated carcinoma, as well as histologically normal ducts *(36,38)*. Thus, the MCF10AT system provides a transplantable xenograft model of human proliferative breast disease with proven neoplastic potential.

Cell Lines and Cell Culture

These studies utilized MCF10A, MCF10AneoT, and MCF10AT3c, a third transplant generation of the MCF10AT xenograft model for human proliferative breast disease. MCF10A cells are ER negative and were used in some experiments to determine the specificity of xenoestrogens for ER-mediated pathways. MCF10AneoT cells and transplant generations derived from MCF10AneoT express functional ER *(39)*. MCF10A and MCF10AT cells were maintained in phenol red-free Dulbecco's modified Eagle medium (DMEM)–F-12 medium with 0.1 μg/mL cholera toxin, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.02 μg/mL epidermal growth factor, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5% horse serum. The human breast carcinoma cell line MCF-7 was maintained in DMEM–F-12 medium supplemented with 10 μg/mL insulin and 5% calf serum. Charcoal-stripped serum was not used in cultures of MCF-7 or MCF10A/MCF10AT cells, since it reduced the proliferative capacity and/or viability of MCF10A/MCF10AT and MCF-7 cells, possibly because of removal of essential growth factors *(40)*. However, since the studies examined estrogenicity of organochlorines, the only sera used routinely were those that were unable to support growth of the ER-positive cell line MCF-7, indicating absence of biologically significant levels of 17β-estradiol (E_2) or other estrogenic compounds.

Cell Proliferation Assay

To assess growth effects of DDT and PCB compounds, we estimated the cell number at the end of a 10-day treatment. Cells were seeded at 5×10^4 (MCF-7) or 1×10^4 (MCF10AneoT or MCF10AT3c) cells in 25-cm^2 flasks. Cells were plated in the appropriate medium as described above; on the following day, treatments were begun with a change of medium twice a week. Treatment consisted of vehicle (ethanol or dimethyl sulfoxide [DMSO] at 0.01%), E_2 (1 or 10 n*M*), *o,p'*-DDT, *p,p'*-DDT, Heptachlor, Aroclor 1221, and Aroclor 1254. All PCB compounds were tested individually at 1 or 10 μ*M; p,p'*-DDT was tested at 0.01, 0.1, 1, and 10 μ*M* concentrations. The influence of combinations of *o,p'*-DDT–*p,p'*-DDT and Aroclor 1221–Aroclor 1254 on cell proliferation was tested at 0.1, 1, and 10 μ*M* concentrations. The pure antiestrogen ICI 182,780, a gift from A. Wakeling (Zeneca Pharmaceuticals, Cheshire, U.K.), was included in the treatment with E_2 or xenoestrogens at 100-fold molar excess of the ligand tested. At the end of 10 days, monolayers were washed with saline and lysed in 0.5 mL 0.01 *M* Hepes buffer–1.5 m*M* $MgCl_2$ plus two drops of ZAP reagent (Acros Organics USA, Pittsburgh, PA) for 5 minutes. The nuclei released were counted in a Coulter counter (Coulter Corp., Miami, FL). All cell counts were done from triplicate flasks, and the results were expressed as the means ± standard error (SE) from three independent experiments.

Growth in Soft Agar

MCF10AT3c cells (4×10^4) were seeded in 2 mL of 0.3% agar in phenol red-free DMEM–F-12-supplemented medium. This suspension was layered over 1 mL of 0.9% agar medium base layer in 35-mm dishes (Corning Costar Corp., Cambridge, MA) and overlaid with 2 mL of phenol red-free DMEM–F-12 medium containing E_2 (1 or 10 n*M*) or 1 or 10 μ*M* of the individual xenoestrogen (*o,p'*-DDT, *p,p'*-DDT, Heptachlor, Aroclor 1221, or Aroclor 1254). Control dishes were treated with an equivalent concentration of vehicle (ethanol or DMSO to final concentration of 0.01%). All dishes were incubated at 37 °C in 5% CO_2–95% O_2 for 3 weeks with twice weekly medium changes and hormone supplementation. All cultures were examined 24 hours after plating, and cell aggregates that might bias final results were marked. Plates with more than 10 aggregates were discarded. Colony-forming efficiency was calculated by dividing the number of colonies larger than 50 μm in diameter (sized using a calibrated ocular grid) by the number of cells seeded. Ten microscopic fields were counted to calculate the total number of colonies per well for the whole well; reported values are the average count from triplicate wells. The numbers of colonies in different size ranges (50–100 μm, 100–200 μm, 200–300 μm, and >300 μm) were calculated in the same manner.

ER-Binding Assay

MCF-7 cells were grown in monolayer culture in phenol red-free medium with 5% calf serum as described before. Cells were allowed to grow through two doublings to deplete steroid hormone levels in the cells. Cells were then harvested, pelleted, and homogenized in 8 volumes of buffer (10 m*M* Tris–HCl, 2 m*M* dithiothreitol, 1 m*M* EDTA, 10% [vol/vol] glycerol, and 0.5 *M* NaCl [pH 7.4]) at 4 °C. Cell homogenates were centrifuged at 105 000*g* for 1 hour at 4 °C, and resulting high-speed cytosols were stored in aliquots at –70 °C. The ability of xenoestrogens to compete with E_2 for binding to ER was determined in single-point competition assays by the standard dextran-coated charcoal method *(41)*. Cytosolic protein (100 μg) was incubated with 2 n*M* [2,4,6,7-^{3}H]E_2 (110 Ci/mmol; Amersham Corp., Arlington Heights, IL) and increasing amounts of unlabeled diethylstilbestrol (DES) (onefold to 1000-fold excess) or various xenoestrogens (10-fold to 1000-fold excess). Reaction mixtures were incubated at 4 °C for 18 hours. Bound and free radiolabels were separated by the addition of dextran-coated charcoal followed by centrifugation at 10 000*g* for 10 minutes at 4 °C. The amount of specifically bound tracer was expressed as percent of maximal specific binding. Protein assays were performed on the cytosols by the Bradford method *(42)*.

Transient Transfection Assay Using ERETKCAT Construct

Reporter plasmid construct. The estrogenicity of organochlorine compounds was assessed by measuring their effect on transient expression of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene in JA-12, a plasmid with CAT expression regulated by tandem estrogen response elements (ERE) upstream of a minimal thymidine kinase (TK) promoter construct, p-37TKCAT *(43,44)*.

Cell culture and transfection. MCF-7, MCF10A, or MCF10AneoT cells (5×10^5/60-mm dish) were plated in phenol red-free media as described above and maintained for 18 hours prior to transfection with 10 μg of JA-12 plasmid DNA by use of the calcium phosphate method *(45)*. Six hours after transfection, cells were shocked with glycerol (3-minute incubation with 15% glycerol for MCF10A/MCF10AneoT cells or 3-minute incubation with 10% glycerol for MCF-7 cells), washed, and cultured in phenol red-free DMEM–F-12 medium containing E_2 (positive control), *o,p'*-DDT, *p,p'*-DDT, Heptachlor, Aroclor 1221, Aroclor 1254, and/or ICI 182,780 at indicated concentrations for 40 hours. Control cultures were treated with vehicle, ethanol, or DMSO at a final concentration of 0.01%.

CAT assay. Transfected cells were lysed with four cycles of freezing and thawing in 0.25 *M* Tris–HCl (pH 7.5). CAT assays were carried out with reaction mixtures containing 100 μg of supernatant protein and 0.1 μCi of [^{14}C]chloramphenicol (40–60 mCi/mmol; ICN Radiochemicals, Irvine, CA) *(46)*. The assay tubes were incubated for 4 hours at 37 °C. Acetylated chloramphenicol was separated by thin-layer chromatography and quantitated by liquid scintillation counting. The lacZ reporter plasmid pCH110 (Pharmacia, Piscataway, NJ) was cotransfected in some experiments to monitor transfection efficiency and assayed according to the manufacturer's protocol. β-Galactoside standard and *o*-nitrophenyl β-galactoside were purchased from Boehringer Mannheim (Indianapolis, IN).

pS2 Gene Expression and Its Regulation by *o,p'*-DDT and *p,p'*-DDT

The pS2 gene is an endogenous gene regulated by estrogen. Its expression was studied in MCF-7 and MCF10AT3c cells. The cells were grown for 2 days in 75-cm^2 culture flasks in phenol red-free media as described above, followed by a change to the same medium but containing the appropriate ligand additions for a further 96 hours in the case of MCF10AT3c cells or 24 hours in the case of MCF-7 cells. Total cellular RNA was prepared from the cells, and 20-μg aliquots of RNA from MCF-7 cells and 40-μg aliquots of RNA from MCF10AT3c cells were subjected to northern blot analysis in 1.5% agarose–formaldehyde gels. The nylon membranes were hybridized with ^{32}P-labeled complementary DNA (cDNA) for pS2 (gift from Dr. P. Chambon, Institut de la Santé et de la Recherche Médicale, Strasbourg, France). Membranes were washed with 0.5×

SSC (1× SSC = 0.15 *M* NaCl and 0.015 *M* sodium citrate [pH 7.0]) and subjected to autoradiography. Loading was checked by reprobing the blot with 36B4 cDNA *(47)*. Results were quantitated with a model 300A densitometer (Molecular Dynamics, Sunnyvale, CA), and the 36B4 band intensities were used to normalize for equal sample loading.

Data Analysis

With the exception of the binding experiment, data were analyzed with an analysis of variance. In cell proliferation assays, cell counts were transformed by use of the natural logarithm. Specific differences between treatments were examined by use of Student's *t* test. Bonferroni's technique was used to compensate for multiple comparisons, and *t* statistics less than .05 were considered significant. For the binding experiment, we fit linear regressions for each treatment. A *t* test was used to compare the slope for DES, *o,p'*-DDT, *p,p'*-DDT, Heptachlor, Aroclor 1221, or Aroclor 1254 and dexamethasone (control). All *P* values were two-sided.

Results

Effect of DDT and PCB Derivatives on Growth of ER-Positive Human Preneoplastic and Breast Cancer Cells

We monitored the effects of xenoestrogens on cellular proliferation of ER-positive MCF-7, MCF10AneoT, and MCF10AT3c cells [*(39);* Shekhar PVM, Chen ML, Werdell J, Heppner GH, Miller FR, Christman JK: manuscript submitted for publication]. Contrary to previous reports *(28)*, results presented in Table 1 show that, at equivalent doses, *p,p'*-DDT was more potent than *o,p'*-DDT at stimulating proliferation of MCF-7 cells. At a concentration of 10 μ*M*, *p,p'*-DDT was approximately two times more effective than *o,p'*-DDT. Added individually, although a concentration of 0.1 μ*M* *p,p'*-DDT stimulated a significant increase in growth over that of cultures treated with vehicle only ($P = .03$ as determined by the *t* test), a significant dose-dependent induction of growth is observed only at concentrations greater than 1 μ*M* with either *o,p'*-DDT ($P = .001$) or *p,p'*-DDT ($P = .0003$). However, when MCF-7 cells were exposed to combinations of *o,p'*- and *p,p'*-DDT, doses as low as 0.1 μ*M* of each compound were sufficient to elicit cellular proliferation to the same extent as 10 μ*M* of *p,p'*-DDT added singly (Table 1). The measured cumulative effects of *o,p'*-DDT and *p,p'*-DDT on growth were not significantly different from those obtained with each isomer separately as determined by Student's *t* test. This result suggests that the cumulative effect of the two DDT isomers was additive. The majority of growth stimulation induced by *p,p'*-DDT was mediated through the ER-dependent pathway, since the proliferative effect was abolished by a 100-fold molar excess of pure antiestrogen, ICI 182,780. We found that concentrations of 10 μ*M* of the PCBs Aroclor 1221 and Aroclor 1254 induced a significant increase in cell yields over that of control cultures of MCF-7 cells ($P = .008$ and $P = .006$, respectively) when used individually (Table 2). However, mixtures of Aroclor 1221 and Aroclor 1254, even at concentrations as high as 10 μ*M*, failed to elicit a cumulative effect on growth of MCF-7 cells (Table 2). The PCB Heptachlor was ineffective in stimulating growth of MCF-7 cells over that of control cultures (Table 2). We have not been able to demonstrate reproducibly the effects of E_2 or any of the organochlorines on monolayer growth of MCF10AneoT or MCF10AT3c cells.

Table 1. Effect of *o,p'*-DDT and *p,p'*-DDT on growth of MCF-7 human breast cancer cells

Compound (concentration)	Cell No. × 10^{-5}*
None	2.27 ± 0.2
17β-Estradiol (1 n*M*)	4.87 ± 0.2[a]
17β-Estradiol (1 n*M*) + ICI 182,780 (100 n*M*)	2.64 ± 0.1
o,p'-DDT	
(1 μ*M*)	3.02 ± 0.5[b]
(10 μ*M*)	3.79 ± 0.3[c]
p,p'-DDT	
(0.01 μ*M*)	2.75 ± 0.5
(0.1 μ*M*)	2.88 ± 0.5[d]
(1 μ*M*)	3.90 ± 0.04[e]
(10 μ*M*)	6.42 ± 0.4[a]
p,p'-DDT + *o,p'*-DDT	
(0.1 μM each)	6.69 ± 0.2[a]
(1 μM each)	7.06 ± 0.2[a]
p,p'-DDT + *o,p'*-DDT (1 μ*M* each) + ICI 182,780 (100 μ*M*)	2.81 ± 0.1

*Results obtained from three independent experiments performed in triplicate are expressed as means ± standard error. Because no differences in cell numbers were observed in control cultures exposed to ethanol or dimethyl sulfoxide, results are grouped together. Superscript letters indicate doses of compounds that increased cell number significantly over the non-hormone-treated control ([a]$P = .0001$; [b]$P = .02$; [c]$P = .001$; [d]$P = .03$; [e]$P = .0003$). Cumulative effects of mixtures of *o,p'*-DDT and *p,p'*-DDT are additive ($P = .05$).

Effect of DDTs and PCBs on Anchorage-Independent Growth of MCF10AT3c Cells

It has been previously shown that monolayer growth of MCF10A cells and MCF10A cells rendered ER positive after stable transfection with an ER expression plasmid is not enhanced by addition of E_2 to the growth medium *(48)*. MCF10A cells do not form colonies in soft agar *(49)*. However, both transfection with T24 Ha-ras *(37)* and successive transplant passage in nude mice result in enhanced capacity for anchorage-independent growth. Under the conditions of the assay used in our experiments, in the absence of E_2, MCF10AT3c cells had a

Table 2. Effect of polychlorinated biphenyls (PCBs) on estrogen-stimulated growth of MCF-7 human breast cancer cells

Compound (concentration)	Cell No. × 10^{-5}*
None	1.48 ± 0.2
17β-Estradiol (1 n*M*)	4.24 ± 0.5[a]
17β-Estradiol (1 n*M*) + ICI 182,780 (100 n*M*)	1.40 ± 0.1
Heptachlor (10 μ*M*)	1.90 ± 0.1
Aroclor 1221 (10 μ*M*)	2.58 ± 0.3[b]
Aroclor 1254 (10 μ*M*)	2.21 ± 0.1[c]
Aroclor 1221 + Aroclor 1254	
(0.1 μ*M* each)	2.75 ± 0.3
(1 μ*M* each)	2.20 ± 0.2
(10 μ*M* each)	2.84 ± 0.2

*Effects of all three PCBs on growth of MCF-7 cells were tested at 1 and 10 μ*M*; however, results at only 10 μ*M* are shown. Results obtained from three independent experiments performed in triplicate are expressed as means ± standard error. Because no differences in cell numbers were observed in control cultures exposed to ethanol or dimethyl sulfoxide, results are grouped together. Superscript letters indicate compounds that enhanced cell yields significantly over the non-hormone-treated control cultures ([a]$P = .001$; [b]$P = .008$; [c]$P = .006$). Mixtures of Aroclor 1221 and Aroclor 1254 did not elicit significant cumulative effects on cell growth at any of the three concentrations tested.

colony-forming efficiency of 25%, and approximately 40% of these colonies had a diameter greater than 100 μm; none had a diameter greater than 200 μm. The colony-forming efficiency of MCF10AT3c cells dramatically increased after exposure to physiologic concentrations of E_2 (Fig. 1). E_2 treatment also enhanced the rate of growth in soft agar with approximately 7% of colonies larger than 200 μm. Exposure of cells to *o,p'*-DDT, *p,p'*-DDT, and Heptachlor also induced increases in colony-forming efficiency and proliferative effects (Fig. 1), whereas Aroclor 1221 and Aroclor 1254 exerted only a modest increase over that of control (data not shown). No colonies over 200 μm were found in *o,p'*-DDT-treated plates. Although *p,p'*-DDT and Heptachlor enhanced colony growth, formation of colonies over 300 μm was realized only with E_2 or *p,p'*-DDT (Fig. 1).

Regulation of Reporter Gene Expression From a Transiently Transfected ERETKCAT Construct in MCF-7 and MCF10AneoT Cells

Although we have not been able to demonstrate effects of xenoestrogens and E_2 on monolayer growth of MCF10AT system cells, we tested whether binding of DDT or PCB derivatives to ER would induce expression of estrogen-sensitive genes. MCF-7 and MCF10AneoT cells were transiently transfected with an exogenous estrogen-sensitive gene, ERETKCAT, and assayed for the regulatory effects of organochlorines on CAT gene expression. Results of CAT gene expression in MCF-7 cells are shown in Tables 3 and 4 and are consistent with those obtained for MCF-7 cell proliferation assays depicted in Tables 1 and 2. Data in Table 3 confirm that, at equivalent doses, *p,p'*-DDT was significantly more effective than *o,p'*-DDT in regulating transcription from a minimal ERETKCAT plasmid ($P = .0001$) and that concentrations of 10 μ*M p,p'*-DDT were required to invoke inductive effects similar to those of E_2 ($P = .0001$). However, when *o,p'*-DDT and *p,p'*-DDT are mixed together, they induce ER-mediated CAT gene expression at concentrations 10-fold to 100-fold lower than those required when each compound is administered alone ($P = .0001$). Table 3 also shows that *p,p'*-DDT not only could synergize with *o,p'*-DDT but also was able to cooperate synergistically with E_2 (at 10^{-11} *M* or 10^{-10} *M*) to induce ER-mediated CAT gene expression ($P = .0001$). The regulatory effects on CAT gene expression were completely abolished with the estrogen antagonist ICI 182,780 (Table 3). Results of Table 4 show that, although the PCB Heptachlor was as effective as *p,p'*-DDT in regulating transcription of the CAT gene, it did not cooperate synergistically with E_2; in fact, the expression of the CAT gene was lower than that observed in the presence of E_2 or Heptachlor alone (Table 4). These data indicate differences in mechanisms of interaction between *p,p'*-DDT and Heptachlor with E_2 in the cell system used. While a synergism between *p,p'*-DDT and E_2 for ER and the two EREs in the ERETKCAT construct was observed (Table 3), Heptachlor and E_2 appeared to compete rather than to cooperate for these binding sites (Table. 4). Aroclor 1221 and Aroclor 1254 were unable to stimulate ER-driven CAT gene expression from the minimal ERETKCAT plasmid (Table 4).

Table 5 shows that the overall pattern of xenoestrogen-mediated regulation of CAT gene expression in preneoplastic MCF10AneoT cells was similar to that observed in breast cancer MCF-7 cells (Tables 3 and 4) with some cell type differences. The effects of these chlorinated hydrocarbons on CAT gene expression were dependent on the presence of a functional ER, since similar transfection assays performed in ER-negative MCF10A cells did not show ligand-dependent regulation of CAT gene expression (data not shown). Furthermore, xenoestrogen-mediated increases in CAT gene expression were completely abolished by 100-fold molar excess of the pure anties-

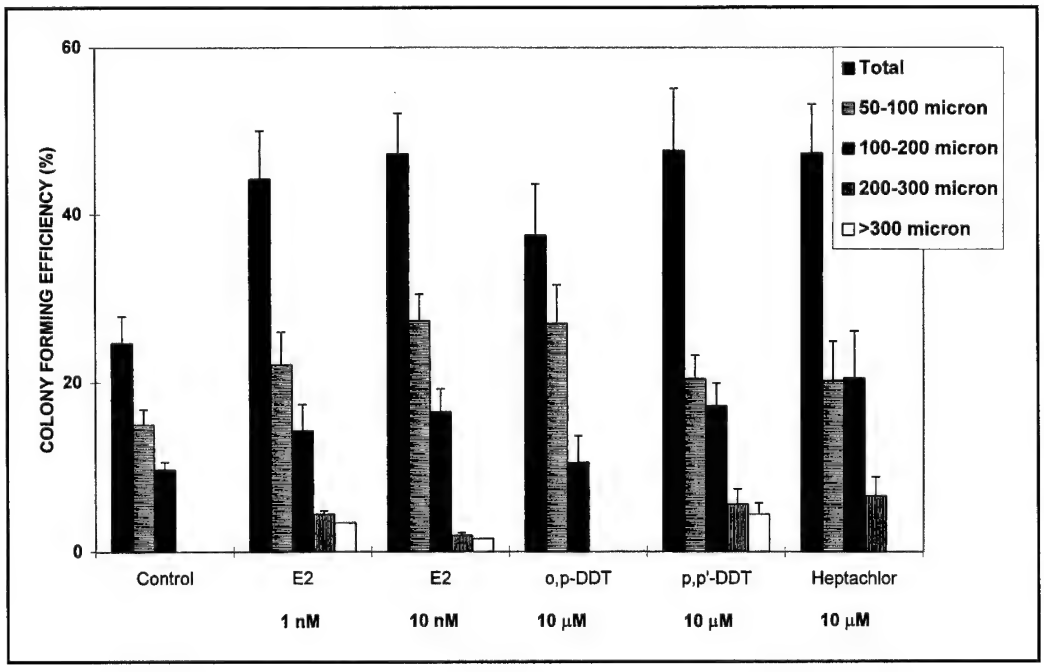

Fig. 1. Effects of DDT and polychlorinated biphenyls (PCBs) on anchorage-independent growth of MCF10AT3c cells. Total number of colonies represent colonies larger than 50 μm in diameter. Colonies were estimated according to their diameter size: small (50–100 μm), medium (100–200 μm), large (200–300 μm), and very large (>300 μm). Control wells received additions of vehicle, ethanol, or dimethyl sulfoxide at a final concentration of 0.01%. Since no differences in colony size or numbers were observed between the two vehicles, results are shown under one group. Treatments included 17β-estradiol (E_2) at a dose of 1 n*M* or 10 n*M*, *o,p'*-DDT at 10 μ*M*, *p,p'*-DDT at 10 μ*M*, and Heptachlor at 10 μ*M*. Colony-forming efficiency was calculated by dividing the number of colonies larger than 50 μm (sized using a calibrated ocular grid) by the number of cells seeded. Ten microscopic fields were counted to calculate the total number of colonies per well for the whole well, and average of three wells for each treatment was calculated. The numbers of colonies in different size ranges (50–100 μm, 100–200 μm, 200–300 μm, and >300 μm) were calculated in the same manner. Results are expressed as means ± standard deviation of three experiments. Where bars are not seen, deviations were too small for visual display.

Table 3. Regulation of CAT gene expression by a mixture of *o,p'*-DDT plus *p,p'*-DDT and *p,p'*-DDT plus 17β-estradiol in ERETKCAT-transfected MCF-7 human breast cancer cells*

Compound (concentration)	% CAT conversion†
None	1.9 ± 0.2
17β-Estradiol	
(0.01 n*M*)	6.8 ± 2.1‡
(0.1 n*M*)	14.3 ± 3.2‡
(1 n*M*)	33.3 ± 4.6‡
17β-Estradiol (1 n*M*) + ICI 182,780 (100 n*M*)	1.4 ± 0.3
o,p'-DDT	
(1 μ*M*)	2.2 ± 0.3
(10 μ*M*)	8.5 ± 0.7‡
p,p'-DDT	
(0.01 μ*M*)	1.2 ± 0.2
(0.1 μ*M*)	4.5 ± 0.3‡
(1 μ*M*)	22.8 ± 3.0‡
(10 μ*M*)	35.1 ± 5.8‡
p,p'-DDT (1 μ*M*) + ICI 182,780 (100 μ*M*)	1.2 ± 0.2
p,p'-DDT (10 μ*M*) + ICI 182,780 (1 m*M*)	3.6 ± 0.2
o,p'-DDT + *p,p'*-DDT	
(0.1 μ*M* each)	47.6 ± 5.7‡
(1 μ*M* each)	50.9 ± 7.8‡
o,p'-DDT + *p,p'*-DDT (1 μ*M* each) + ICI 182,780 (100 μ*M*)	4.7 ± 0.4
17β-Estradiol (0.01 n*M*) + *p,p'*-DDT (1 μ*M*)	50.2 ± 6.9‡
17β-Estradiol (0.1 n*M*) + *p,p'*-DDT (1 μ*M*)	61.9 ± 10.6‡

*CAT = chloramphenicol acetyltransferase; ERE = estrogen response element; TK = thymidine kinase.

†Results obtained from three independent transfections are expressed as means ± standard deviation.

‡Doses of compounds that increased CAT expression significantly over the non-hormone-treated control transfections ($P \leq .001$). CAT expressions for mixtures of *o,p'*-DDT and *p,p'*-DDT on 17β-estradiol and *p,p'*-DDT were significantly different from CAT expressions observed with the individual compounds using Student's *t* test analysis ($P = .0001$). Co-transfection with pCH110, which expresses β-galactosidase activity at a constant level regardless of 17β-estradiol or xenoestrogen concentration, confirmed that the results are not due to differences in transfection efficiency or cell viability (data not shown).

trogen ICI 182,780 (Table 5). Co-transfection with pCH110, which expresses β-galactosidase activity at a constant level regardless of E_2 or xenoestrogen concentration, confirmed that these results were not due to differences in transfection efficiency or cell viability (data not shown).

Regulation of pS2 Gene Expression in Preneoplastic MCF10AT3c and Breast Cancer MCF-7 Cells

To test whether *o,p'*-DDT and *p,p'*-DDT can regulate expression of an endogenous estrogen-regulated gene, expression of pS2 was examined *(50)*. 36B4 was chosen as a control gene, as its expression is not modulated by estrogen and we wanted to ensure equal loading of RNA samples *(46)*. Densitometric analysis of pS2 messenger RNA (mRNA) levels relative to 36B4 message from MCF-7 cells showed that, like E_2 which induced approximately fourfold higher levels of pS2 mRNA, both *o,p'*- and *p,p'*-DDT enhanced approximately threefold and fourfold higher levels of pS2 mRNA (Fig. 2), respectively, when compared with untreated cells that had no detectable pS2 mRNA (data not shown). Although DDT-mediated regulation of pS2 mRNA resulted in induction effects similar to those of E_2, 10

Table 4. Regulation of CAT gene expression by PCBs from ERETKCAT-transfected MCF-7 human breast cancer cells*

Compound (concentration)	% CAT conversion†
None	1.7 ± 0.2
17β-Estradiol (1 n*M*)	33.3 ± 5.8‡
17β-Estradiol (1 n*M*) + ICI 182,780 (100 n*M*)	1.0 ± 0.2
Aroclor 1221	
(1 μ*M*)	1.5 ± 0.3
(10 μ*M*)	1.2 ± 0.4
Aroclor 1254	
(1 μ*M*)	1.3 ± 0.2
(10 μ*M*)	2.9 ± 0.2
Heptachlor (10 μ*M*)	39.2 ± 6.8‡
Heptachlor (10 μ*M*) + 17β-estradiol (1 n*M*)	22.4 ± 4.5

*CAT = chloramphenicol acetyltransferase; PCBs = polychlorinated biphenyls; ERE = estrogen response element; TK = thymidine kinase.

†Results obtained from three independent transfections are expressed as means ± standard deviation.

‡Compounds that increased CAT expression significantly over the non-hormone-treated control transfections ($P = .0001$). Co-transfection with pCH110, which expresses β-galactosidase activity at a constant level regardless of 17β-estradiol or xenoestrogen concentration, confirmed that the results are not due to differences in transfection efficiency or cell viability (data not shown).

Table 5. Regulation of CAT gene expression by DDT and PCBs from ERETKCAT-transfected MCF10AneoT cells*

Compound (concentration)	% CAT conversion†
None	8.5 ± 2.4
17β-Estradiol (1 n*M*)	19.5 ± 1.3‡
17β-Estradiol (1 n*M*) + ICI 182,780 (100 n*M*)	8.6 ± 1.8
o,p'-DDT	
(1 μ*M*)	12.2 ± 1.2‡
(10 μ*M*)	17.4 ± 1.8‡
o,p'-DDT (10 μ*M*) + ICI 182,780 (1 m*M*)	10.9 ± 1.8
p,p'-DDT	
(1 μ*M*)	17.1 ± 4.1‡
(10 μ*M*)	27.6 ± 1.4‡
p,p'-DDT (10 μ*M*) + ICI 182,780 (1 m*M*)	9.2 ± 2.3
Aroclor 1221	
(1 μ*M*)	11.8 ± 1.2
(10 μ*M*)	13.2 ± 2.0‡
Aroclor 1254	
(1 μ*M*)	10.5 ± 2.3
(10 μ*M*)	11.2 ± 3.1
Heptachlor	
(1 μ*M*)	23.8 ± 3.0‡
(10 μ*M*)	40.0 ± 5.4‡
Heptachlor (10 μ*M*) + ICI 182,780 (1 m*M*)	13.4 ± 2.2

*CAT = chloramphenicol acetyltransferase; PCBs = polychlorinated biphenyls; ERE = estrogen response element.

†Results obtained from three independent transfections are expressed as means ± standard deviation.

‡All compounds, except Aroclor 1254, increased CAT expression significantly over the non-hormone-treated control transfections ($P = .001$). Co-transfection with pCH110, which expresses β-galactosidase activity at a constant level regardless of 17β-estradiol or xenoestrogen concentration, confirmed that the results are not due to differences in transfection efficiency or cell viability (data not shown).

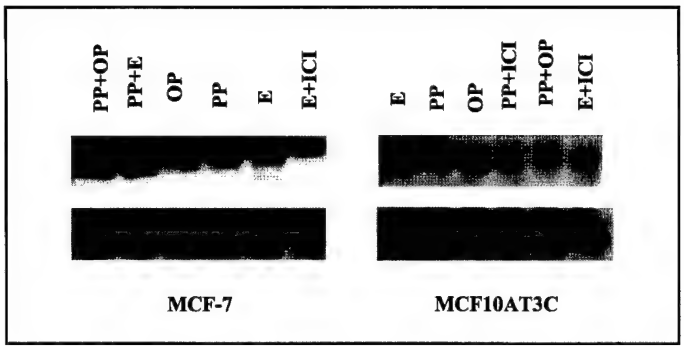

Fig. 2. Regulation by *o,p'*-DDT and *p,p'*-DDT of pS2 messenger RNA in preneoplastic MCF10AT3c and MCF-7 human breast cancer cells. Total cellular RNA isolated at 96 hours from MCF10AT3c (40 μg) and at 24 hours from MCF-7 (20 μg) cells following treatment with 17β-estradiol (E; 1 n*M*), *o,p'*-DDT (OP; 10 μ*M*), *p,p'*-DDT (PP; 10 μ*M*), or mixtures of *p,p'*-DDT and *o,p'*-DDT (PP + OP; 0.1 μ*M* of each ligand) or *p,p'*-DDT (0.1 μ*M*) and 17β-estradiol (1 n*M*) (PP + E) was subjected to northern blot analysis as described in the "Materials and Methods" section. ICI 182,780 (ICI) was added at 100-fold molar excess of 17β-estradiol or *p,p'*-DDT. Blots were probed for expression of pS2, an estrogen-regulated gene (top row), and for 36B4 messenger RNA, a non-estrogen-regulated gene (bottom row), to ensure equal RNA loading. Results were quantitated by densitometric scanning, and the pS2 signals were normalized to 36B4 signals.

μ*M* *o,p'*-DDT or *p,p'*-DDT was required to evoke pS2 stimulation compared with 1 n*M* E_2 (Fig. 2). Treatment of MCF-7 cells with a mixture of *o,p'*-DDT and *p,p'*-DDT at 100-fold lower doses (0.1 μ*M* of each ligand) caused an approximately 10-fold enhancement of pS2 transcription relative to untreated cells (Fig. 2). Similar enhancements in pS2 message levels (about ninefold) were observed when MCF-7 cells were exposed to a mixture of *p,p'*-DDT (0.1 μ*M*) and E_2 (1 n*M*) (Fig. 2). Expression of pS2 was examined in MCF10AT3c cells rather than in MCF10AneoT cells, since more MCF10AT3c cells than MCF10AneoT cells are ER positive (Shekhar PVM, Chen ML, Werdell J, Heppner GH, Miller FR, Christman JK: manuscript submitted for publication). In contrast to MCF-7 cells that showed a rapid increase in accumulation of pS2 transcripts after exposure to E_2 or DDT, induction of pS2 mRNA in MCF10AT3c cells occurred more slowly. Levels of pS2 mRNA were very low in MCF10AT3c cells treated with E_2 or DDT for 24 hours (data not shown); however, pS2 levels rose following exposure of the cells for 4 days (Fig. 2). Although the kinetics of pS2 induction differed greatly in MCF-7 and MCF10AT3c cells, pS2 gene expression was stimulated by E_2 and *p,p'*-DDT by about fourfold relative to untreated cells (which had undetectable pS2 signal; data not shown) in both cell lines. However, unlike MCF-7 cells, treatment of MCF10AT3c cells with mixtures of *o,p'*-DDT and *p,p'*-DDT (0.1 μ*M* of each ligand) induced pS2 mRNA at a level intermediate between induced levels with either *p,p'*-DDT or *o,p'*-DDT alone (approximately twofold; Fig. 2). Addition of antiestrogen, ICI 182,780, at 100-fold molar excess of E_2 or *p,p'*-DDT to treated MCF-7 or MCF10AT3c cells substantially reduced (by about 60%) the levels of pS2 transcripts relative to cells treated with E_2 or *p,p'*-DDT alone (Fig. 2).

Ligand Binding to ER

Since the effects of estrogen are mediated by the binding to its intracellular receptor, we tested the binding ability of DDT and PCB compounds to ER in a single-point competitive binding assay. ER prepared from MCF-7 cells was incubated with [^{3}H]E_2, and the degree of inhibition of binding was determined in the presence of increasing amounts of DDT or PCB compounds. Among the xenoestrogens tested, only *o,p'*-DDT competed with E_2 binding to ER (P = .03). Approximately 40% inhibition of binding was effected by 500-fold molar excess of *o,p'*-DDT, whereas *p,p'*-DDT(P = .35) and Aroclor 1221 (P = .48) caused minimal (<20%) inhibition at similar concentrations (Fig. 3). Heptachlor, Aroclor 1254, and dexamethasone had no effect on [^{3}H]E_2 binding (Fig. 3).

Discussion

The above studies show that, among a series of DDT and PCB compounds tested, the DDT isomer *p,p'*-DDT is the most effective organochlorine in regulating ER-mediated cellular responses in our models of human preneoplastic and breast cancer cells. Besides establishing the estrogenicity of *p,p'*-DDT, we show for the first time that the two isomers of DDT, *o,p'*-DDT and *p,p'*-DDT, can cooperate to induce ER-mediated processes, viz., increase in cell proliferation and transactivation of exogenous (from a minimal ERETKCAT plasmid) and endogenous estrogen-regulated (pS2) genes. We also show for the first time that, whereas *p,p'*-DDT can synergistically collaborate with E_2 to induce ER-mediated increase in CAT gene expression, Heptachlor, an estrogenic PCB, exhibits a competitive interaction with E_2, with the resultant signal being smaller than the signals generated by Heptachlor or E_2 alone. Used individually, con-

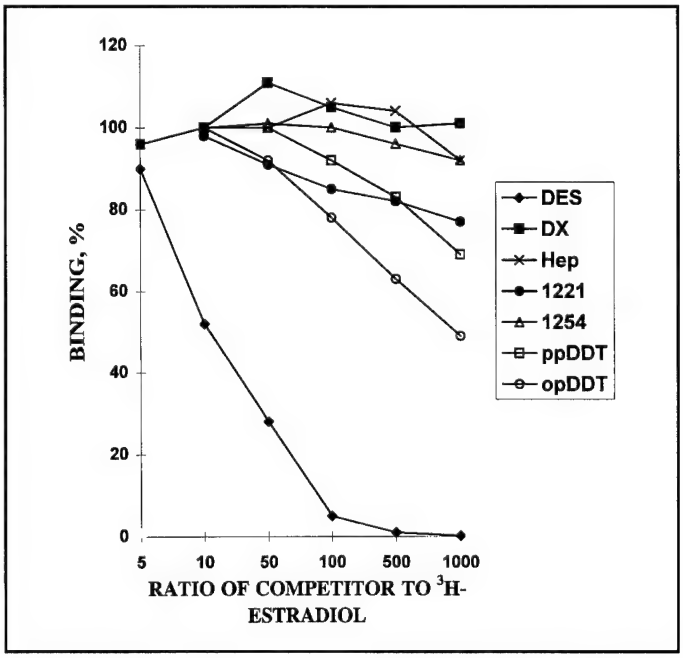

Fig. 3. Binding of DDT isomers and polychlorinated biphenyls (PCBs) to estrogen receptor from MCF-7 human breast cancer cells. 2,4,6,7-[^{3}H]17β-estradiol (2 n*M*) was incubated with cytosol prepared from MCF-7 cells and the indicated molar excess of diethylstilbestrol (DES), *o,p'*-DDT (opDDT), *p,p'*-DDT (ppDDT), Aroclor 1221 (1221), Aroclor 1254 (1254), Heptachlor (Hep), or dexamethasone (DX) in a single-point competitive binding assay as described in the "Materials and Methods" section. Among all the xenoestrogens tested, only *o,p'*-DDT competed with [^{3}H]17β-estradiol binding to estrogen receptor (P = .03).

centrations of 10 μ*M* of the DDT isomers are required for induction of ER-mediated increases in cell proliferation and CAT gene expression; however, effective concentrations of the individual isomers that generate similar magnitudes of ER-mediated cellular responses are dramatically reduced 10-fold to 100-fold when mixtures of the two DDT isomers are used. Although our data show *o,p'*-DDT, *p,p'*-DDT, and Heptachlor to have estrogenic properties, none of these compounds compete as effectively with [^{3}H]E_2 as DES. Consistent with previous reports, our data from competition assays in an *in vitro* cell-free system show *o,p'*-DDT to be most efficient in inhibiting binding of [^{3}H]E_2 *(33,34),* although functional assays using cultured cells show a stronger *p,p'*-DDT-mediated estrogenic effect. Furthermore, whereas xenoestrogens exert pleiotropic effects on cell growth and differentiation via deregulation of signal transduction pathways, the bulk of the growth response induced by these xenobiotics is abolished by the pure antiestrogen ICI 182,780.

DDT isomers are estrogenic in the two systems tested: preneoplastic and breast cancer cells. However, a major difference was observed, viz., a lack of cooperation between these derivatives in the induction of pS2 expression in the preneoplastic breast epithelial cells. Regulation of pS2 gene expression in MCF10AT3c cells by *o,p'*-DDT and *p,p'*-DDT can be explained by a direct competition between the two isomers for binding to the ER, suggesting that both isomers of DDT induce pS2 transcription but at different rates, so that the final levels of pS2 mRNA measured represent the average of the two isomers. This variation in estrogenic response of MCF10AT3c cells may be ascribed to differences in cell type or to differences in ER levels in the two cell lines. Similar differences in synergistic interactions between E_2 and 3,4,3',4'-tetrachlorobiphenyl on pS2 synthesis have been attributed to variations in cell type of two human breast cancer cells that express high levels of ER: MCF-7 versus ZR-75–1 *(51).* Another difference observed in our two human breast cell systems is the lack of an association between estrogenicity of Heptachlor and absence of growth stimulation in MCF-7 cells; in contrast, in MCF10AT cells, Heptachlor is a potent stimulator of both CAT gene expression and colony growth. It is becoming increasingly clear that estrogenic properties (activity and potency) of organochlorines are greatly influenced by the assay system, cell type, level of ER (endogenous or exogenous), number of EREs in the reporter construct, and sensitivity of the assay used. This disparity in estrogenicity of various xenobiotics measured by different assays compel the inclusion of common standards, both positive and negative, to substantiate assays that may provide results most relevant to assessing cancer risk in humans.

Although many xenoestrogens are less potent than biologic estrogens and do not produce mammary gland neoplasms in the bioassay rodent models, their ability to promote growth and proliferation of breast epithelial cells that are at high risk of developing cancer has not been investigated. The daily exposure from xenoestrogenic pesticides such as DDT, dieldrin, endosulfan, and methoxychlor is approximately 2.5 μg *(10,52).* Serum concentration of DDT in women range from 2 to 15 n*M (53–56),* and a recent report by Zava et al. *(57)* using an *in vitro* cell culture system has shown that the concentration of *o,p'*-DDT required to half-saturate ER is about 1000-fold higher than the reported levels of DDT in serum *(53–56).* While these data would suggest that DDT and other xenoestrogens influence estrogen-mediated cellular responses only minimally, xenoestrogens are still cause for concern because of their persistence in the environment, resistance to enzymatic or chemical degradation, sequestration and storage by adipose tissues, and long half-life *(7).* In fact, the levels of xenoestrogens, particularly those of DDT, detected in adipose tissue of human breast cancer are nearly 1000-fold higher than their levels in serum and are parallel with the concentrations required for *in vitro* stimulation of cells, suggesting that the doses (1–10 μ*M*) are indeed physiologically relevant *(53–58).* A detailed comparison of organochlorine pesticide residues collected in samples of maternal blood, milk, subcutaneous fat, and umbilical cord showed the presence of a variety of organochlorines *(59).* The major pesticide residues detected in all samples were *p,p'*-DDT and *p,p'*-DDE, with small amounts of *o,p'*-DDT and significantly smaller amounts of β-hexachlorocyclohexane and dieldrin *(59).* Since the ban on usage of DDT and restricted usage of PCBs in the United States, exposures to these compounds have been so drastically reduced that they have been considered to pose a negligible health risk. However, the adverse effects of these organochlorines could be overlooked if attention is paid only to tissue levels since, through cooperation with other xenoestrogens or endogenous weak estrogens as our data and previous reports *(60)* show, these organochlorines could produce significant estrogenic effects. About 40% of all cancers in women are hormonally mediated *(61),* and epidemiologic evidence suggests that a major determinant of nongenetic breast cancer risk is total cumulative exposure to estrogen *(62,63).* Reports on ER-mediated cellular responses of organochlorines in human breast cancer cell lines necessitate the evaluation of the role of xenoestrogens *in vivo* in breast carcinogenesis *(60,64).* A detailed follow-up of breast cancer patients with high levels of DDT and other xenoestrogens and of patients with ER-positive breast tumors who fail to respond to endocrine intervention therapies is crucial to establish whether xenoestrogens contribute to breast cancer risk and incidence.

References

(1) Hunter DJ, Kelsey KT. Pesticide residues and breast cancer: the harvest of a silent spring? [editorial]. J Natl Cancer Inst 1993;85:598–9.

(2) Colborn T, Vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 1993;101:378–84.

(3) Rennie J. Malignant mimicry. False estrogens may cause cancer and lower sperm counts [news]. Sci Am 1993;269:34.

(4) Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 1993;341: 1392–5.

(5) Sumpter JP, Jobling S. Male sexual development in "a sea of oestrogen" [letter]. Lancet 1993;342:124–5.

(6) Davis DL, Bradlow HL, Wolff M, Woodruff T, Hoel DG, Anton-Culver H. Medical hypothesis: xenoestrogens as preventable causes of breast cancer. Environ Health Perspect 1993;101:372–7.

(7) Stone R. Environmental estrogens stir debate. Science 1994;265:308–10.

(8) Safe SH. Dietary and environmental estrogens and antiestrogens and their possible role in human disease. Environ Sci Pollut Res 1994;1:29–33.

(9) Fagin D. Estrogen link. Newsday 1994 (28 Dec), A7-A9.

(10) Safe SH. Environmental and dietary estrogens and human health: is there a problem? Environ Health Perspect 1995;103:346–51.

(11) Davidson NE, Lippman ME. The role of estrogens in growth regulation of breast cancer. Crit Rev Oncog 1989;1:89–111.

(12) Weisz A, Bresciani F. Estrogen regulation of proto-oncogenes coding for nuclear proteins. Crit Rev Oncog 1993;4:361–88.
(13) Weisz A, Rosales R. Identification of an estrogen response element upstream of the human c-fos gene that binds the estrogen receptor and the AP-1 transcription factor. Nucleic Acids Res 1990;18:5097–106.
(14) Hyder SM, Stancel GM, Nawaz Z, McDonnell DP, Loose-Mitchell DS. Identification of an estrogen response element in the 3′-flanking region of the murine c-fos protooncogene. J Biol Chem 1992;267:18047–54.
(15) Dubik D, Shiu RP. Mechanism of estrogen activation of c-myc oncogene expression. Oncogene 1992;7:1587–94.
(16) Cicatiello L, Ambrosino C, Coletta B, Scalona M, Sica V, Bresciani F, et al. Transcriptional activation of jun and actin genes by estrogen during mitogenic stimulation of rat uterine cells. J Steroid Biochem Mol Biol 1992;41:523–8.
(17) Cicatiello L, Sica V, Bresciani F, Weisz A. Identification of a specific pattern of "immediate-early" gene activation induced by estrogen during mitogenic stimulation of rat uterine cells. Receptor 1993;3:17–30.
(18) Hyder SM, Nawaz Z, Chiappetta C, Yokoyama K, Stancel GM. The proto-oncogene c-jun contains an unusual estrogen-inducible enhancer within the coding sequence. J Biol Chem 1995;270:8506–13.
(19) Jensen AA. Environmental and occupational chemicals. In: Bennet PN, editor. Drugs and human lactation. New York: Elsevier Sci Publ, 1988:551–73.
(20) Kutz FW, Wood PH, Bottimore DP. Organochlorine pesticides and polychlorinated biphenyls in human adipose tissue. Rev Environ Contam Toxicol 1991;120:1–82.
(21) Wolff MS. Occupationally derived chemicals in breast milk. Am J Ind Med 1983;4:259–81.
(22) Mussalo-Rauhamaa H. Partitioning and levels of neutral organochlorine compounds in human serum, blood cells, and adipose and liver tissue. Sci Total Environ 1991;103:159–75.
(23) Rogan WJ, Gladen BC, McKinney JD, Carreras N, Hardy P, Thullen J, et al. Polychlorinated biphenyls (PCBs) and dichlorodiphenyl dichloroethene (DDE) in human milk: effects of maternal factors and previous lactation. Am J Public Health 1986;76:172–7.
(24) Levine R. Recognized and possible effects of pesticides in humans. In: Hayes WJ Jr, Laws ER Jr, editors. Handbook of pesticide toxicology. Vol 1. General principles. San Diego:Academic Press, 1991:275–360.
(25) Safe S. Toxicology, structure–function relationship, and human and environmental health impacts of polychlorinated biphenyls: progress and problems. Environ Health Perspect 1993;100:259–68.
(26) International Agency for Research on Cancer. Overall evaluations of carcinogenicity: an update of IARC Monographs Volumes 1 to 42. Geneva: WHO, 1987:186–7.
(27) Smith AG. Chlorinated hydrocarbon insecticides. In: Hayes WJ Jr, Laws ER Jr, editors. Handbook of pesticide toxicology. Vol 2. Classes of pesticides. San Diego: Academic Press, 1991:731–915.
(28) Welch RM, Levin W, Conney AH. Estrogenic action of DDT and its analogs. Toxicol Appl Pharmacol 1969;14:358–67.
(29) Kupfer D, Bulger WH. Estrogenic properties of DDT and its analogs. In: McLachlan J, editor. Estrogens in the environment. New York: Elsevier, 1980:239–63.
(30) Bulger WH, Kupfer D. Estrogenic action of DDT analogs. Am J Ind Med 1983;4:163–73.
(31) Scribner JD, Mottet NK. DDT acceleration of mammary gland tumors induced in the male Sprague-Dawley rat by 2-acetamidophenanthrene. Carcinogenesis 1981;2:1235–9.
(32) Robison AK, Sirbasku DA, Stancel GM. DDT supports growth of an estrogen-responsive tumor. Toxicol Lett 1985;27:109–13.
(33) Mason RR, Schulte GJ. Interaction of *o,p′*-DDT with the estrogen-binding protein (EBP) of DMBA-induced rat mammary tumors. Res Commun Chem Pathol Pharmacol 1981;33:119–28.
(34) Kupfer D, Bulger WH. Interaction of *o,p′*-DDT with the estrogen-binding protein (EBP) in human mammary and uterine tumors. Res Commun Chem Pathol Pharmacol 1977;16:451–62.
(35) Soto AM, Lin TM, Justicia H, Silvia RM, Sonnenscheim C. An "in culture" bioassay to access the estrogenicity of xenobiotics (E. screen). In: Colbom T, Clemens C, editors. Chemically induced alterations in sexual and functional development: the wild-life/human connection. Advances in modern environmental toxicology. Vol 21. Princeton (NJ): Princeton Scientific Publishing, 1992:107–12.
(36) Miller FR, Soule HD, Tait L, Pauley RJ, Wolman SR, Dawson PJ, et al. Xenograft model of human proliferative breast disease. J Natl Cancer Inst 1993;85:1725–32.
(37) Basolo F, Elliott J, Tait L, Chen XQ, Maloney T, Russo IH, et al. Transformation of human breast epithelial cells by c-Ha-ras oncogene. Mol Carcinog 1991;4:25–35.
(38) Dawson PJ, Wolman SR, Tait L, Heppner GH, Miller FR. MCF10AT: a model for the evolution of cancer from proliferative breast disease. Am J Pathol 1996;148:313–9.
(39) Shekhar PV, Chen ML, Werdell J, Heppner GH, Miller FR, Christman JK. Activation of the endogenous estrogen receptor gene in MCF10AT system cells, a potential factor for neoplastic progression of MCF10AT xenografts. Proc Am Assoc Cancer Res 1995;36:255.
(40) Wiese TE, Kral LG, Dennis KE, Butler WB, Brooks SC. Optimization of estrogen growth response in MCF-7 cells. In Vitro Cell Dev Biol 1992; 28A:595–602.
(41) Green B, Leake RE. Steroid hormones: a practical approach. Oxford (U.K.): IRL Press, 1987.
(42) Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
(43) VanderKuur JA, Hafner MS, Christman JK, Brooks SC. Effects of estradiol-17β analogues on activation of estrogen response element regulated chloramphenicol acetyltransferase expression. Biochemistry 1993:32: 7016–21.
(44) Strahle U, Schmid W, Schutz G. Synergistic action of the glucocorticoid receptor with transcription factors. EMBO J 1988;7:3389–95.
(45) Graham FL, Eb AJ van der. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 1973;52:456–67.
(46) Gorman CM, Moffat LF, Howard BH. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 1982;2:1044–51.
(47) Brown AM, Jeltsch JM, Roberts M, Chambon P. Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. Proc Natl Acad Sci U S A 1984;81:6344–8.
(48) Pilat MJ, Christman JK, Brooks SC. Characterization of the estrogen receptor transfected MCF10A breast cell line 139B6. Breast Cancer Res Treat 1996;37:253–66.
(49) Soule HD, Maloney TM, Wolman SR, Peterson WD Jr, Brenz R, McGrath CM, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 1990;50: 6075–86.
(50) Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P. Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. Nucleic Acids Res 1982;10:7895–903.
(51) Nesaretnam K, Corcoran D, Dils RR, Darbre P. 3,4,3′,4′-Tetrachlorobiphenyl acts as an estrogen *in vitro* and *in vivo*. Mol Endocrinol 1996; 10:923–6.
(52) Safe SH. Do environmental estrogens play a role in development of breast cancer in women and male reproductive problems? Hum Ecol Risk Assess 1995;1:17–23.
(53) MacMahon B. Pesticide residues and breast cancer? [editorial]. J Natl Cancer Inst 1994;86:572–3.
(54) Dewailly E, Dodin S, Verreault R, Ayotte P, Sauve L, Morin J, et al. High organochlorine body burden in women with estrogen receptor-positive breast cancer. J Natl Cancer Inst 1994;86:232–4.
(55) Krieger N, Wolff MS, Hiatt RA, Rivera M, Vogelman J, Orentreich N. Breast cancer and serum organochlorines: a prospective study among white, black, and Asian women. J Natl Cancer Inst 1994;86: 589–99.
(56) Djordjevic MV, Hoffmann D, Fran J, Prokopczyk B, Citron ML, Stellman SD. Assessment of chlorinated pesticides and polychlorinated biphenyls in adipose breast tissue using a supercritical fluid extraction method. Carcinogenesis 1994;15:2581–5.
(57) Zava DT, Blen M, Duwe G. Estrogenic activity of natural and synthetic estrogens in human breast cancer cells in culture. Environ Health Perspect 1997;105 Suppl 3:637–45.
(58) Wolff MS, Toniolo PG, Lee EW, Rivera M, Dubin N. Blood levels of organochlorine residues and risk of breast cancer. J Natl Cancer Inst 1993; 85:648–52.

(59) Kanja LW, Skaare JU, Ojwang SB, Maitai CK. A comparison of organochlorine pesticide residues in maternal adipose tissue, maternal blood, cord blood and human milk from mother/infant pairs. Arch Environ Contam Toxicol 1992;22:21–4.
(60) Soto AM, Chung KL, Sonnenschein C. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ Health Perspect 1994;102:380–3.
(61) Henderson BE, Ross RK, Pike MC. Toward the primary prevention of cancers. Science 1991;254:1131–8.
(62) Lipworth L. Epidemiology of breast cancer. Eur J Cancer Prev 1995;4: 7–30.
(63) Miller WR. Endocrine treatment for breast cancers: biological rationale and current progress. J Steroid Biochem Mol Biol 1990;37:467–80.
(64) Steinmetz R, Young PC, Caperell-Grant A, Gize EA, Madhukar BV, Ben-Jonathan N, et al. Novel estrogenic action of the pesticide residue β-hexachlorocyclohexane in human breast cancer cells. Cancer Res 1996;56: 5403–9.

Notes

Supported by Public Health Service grants CA60881 (P. V. M. Shekhar) and CA22453 (Cancer Center Support grant to the Karmanos Cancer Institute) from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services; and by the U.S. Army Medical Research and Materiel Command (DAMD17-94-J-4427).

We thank Dr. Raymond Novak and Dr. Tom Kocarek for generously providing all the xenobiotics used in this study and Dr. Phyllis Gimotty for statistical analysis of data. We also thank Dr. P. Chambon and Dr. A. Wakeling for their generous gifts of human pS2 complementary DNA and ICI 182,780, respectively. We gratefully acknowledge Dr. Gloria Heppner's critical input and valuable suggestions.

Manuscript received April 10, 1997; revised July 16, 1997; accepted September 17, 1997.

INTERNATIONAL JOURNAL OF ONCOLOGY 11: 1087-1094, 1997

Altered P53 conformation: A novel mechanism of wild-type *p53* functional inactivation in a model for early human breast cancer

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Received July 3, 1997; Accepted August 22, 1997

Abstract. The p53 protein is a transcription factor that is frequently mutated in human malignancies. Using the MCF10AT model for early human breast cancer we show that P53 protein is unmutated indicating that mutations are not necessary for alterations in growth and morphology that accompany preneoplastic stages of breast tumor progression. Although p53 protein is wild-type in cells of the MCF10AT model system, it exists predominantly in a conformationally altered state that is defective in its ability both to bind DNA in a sequence-specific manner and to induce transcriptional activation from the WAF-1 promoter. This contrasts with P53 from the non-tumorigenic parental MCF10A cells which is predominantly conformationally normal and functionally active. The possibility that stabilized wild-type but conformationally altered P53 plays a role in the neoplastic progression of preneoplastic MCF10AT system cells is discussed.

Introduction

Inactivation of the *p53* tumor suppressor gene is one of the most common events in neoplastic transformation. Point mutations or other genomic alterations in *p53* have been identified in more than 50% of human cancers. In many of the remaining tumor types, wild-type P53 protein is functionally inactivated by mechanisms that include binding to the cellular MDM2 oncoprotein or sequestering of P53 in the cytoplasm (1). While missense mutations of *p53* gene represent an early event in the transformation and progression of tumors of the colon (2), esophagus (3) and hematopoietic system (4), mutations of *p53* in breast cancer are less common, occurring in 20-30% of breast carcinomas examined (5).

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Key words: MCF10A, T24 Ha-*ras*, xenograft model, conformationally altered wild-type P53, preneoplasia

Structurally, P53 is a typical transcription factor containing identifiable transcription activation, sequence-specific DNA binding and oligomerization domains (6). Mutational analysis has identified all three of these domains as essential for the growth suppressive properties of P53, i.e., deletion of any one of these domains inhibits the ability of P53 to act as a tumor suppressor (7). Wild-type P53 recognizes a 20 bp motif consisting of two copies of the consensus sequence 5'-PuPuPuC(T/A)(T/A)GPyPyPy-3'. When these P53-responsive elements are adjacent to a minimal promoter they stimulate gene expression in a P53-dependent fashion (8-10). Mutant P53 proteins that fail to bind DNA (8) also fail to act as transcriptional activators at P53-response elements (11-13). Muscle creatine kinase, MDM2, GADD45, WAF1/Cip1, epidermal growth factor receptor and cyclin G genes (14-19) are among the genes that contain sites with high homology to the 20 bp P53 binding site consensus motif. P53 plays a role in regulating genes involved in cell cycle (20), cell cycle arrest after DNA damage (16), and commitment of some cells to apoptosis (21-23).

We have utilized the MCF10AT model system to evaluate the importance of P53 alterations in the early stages of breast cancer. This model is comprised of an array of cell lines that undergo a sequence of progressive histological changes mimicking those seen in breasts of women at high risk for breast cancer (24-26). Here, we show that wild-type P53 protein in cells of the MCF10AT system exists predominantly in a conformationally altered state that is defective in its ability both to bind DNA in a sequence-specific manner and to induce transcriptional activation from the WAF-1 promoter. This contrasts with P53 from the non-tumorigenic parental MCF10A cells which is functionally active. The possibility that stabilized wild-type but conformationally altered P53 plays a role in the neoplastic progression of preneoplastic MCF10AT cells is discussed.

Materials and methods

Cell culture. MCF10A, MCF10Aneo (vector transfected), MCF10AneoN (normal Ha-*ras*-transfected), MCF10AneoT (T24 Ha-*ras*-transfected) (24) and cell lines derived from MCF10AneoT (MCF10AT1, T2b, T3b and T3c) (25,26) by

alternating *in vivo* transplantation and *in vitro* culture (MCF10AT system cells) were maintained in DMEM/F-12 medium supplemented with 2.5% equine serum, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.02 μg/ml epidermal growth factor, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Analysis of P53 protein expression

Metabolic labeling. Exponentially growing MCF10A and MCF10A-derived cells were incubated for 3 h in methionine-free DMEM with 2% dialyzed fetal bovine serum supplemented with 100 μCi of [^{35}S]-methionine (specific activity 1,083 Ci/mmol, New England Nuclear, Boston, MA). Monolayers were gently washed twice with phosphate-buffered saline (PBS) and treated with 400 μl of lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM phenyl methyl sulfonyl fluoride, PMSF) at 4°C. Cell lysates were cleared by centrifugation for 15 min at 10,000 x g and used immediately for immunoprecipitation. Aliquots of lysate containing equivalent amounts of radiolabel incorporated into trichloracetic acid insoluble material (10^7 cpm) were immunoprecipitated by incubating overnight at 4°C with 1 μg of anti-P53 monoclonal antibodies (mAbs) pAb421 (27) or pAb240 (28; Oncogene Science, Inc., Cambridge, MA) or normal mouse IgG. Protein G Sepharose beads were then added and incubation continued for 1 h at 4°C. Sepharose beads were pelleted by centrifugation and washed four times in lysis buffer. Bound proteins were resolved by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis on 8.5% polyacrylamide gels. Gels were fixed and processed for fluorography. Radiolabeled proteins were visualized by autoradiography of the dried gels.

Immunofluorescence. MCF10AT3b ($5x10^4$) cells were plated on coverslips in media as described before, and after 2-3 days of culture when the cells reached confluence, the slides were rinsed twice with PBS and fixed in methanol:acetone (1:1) for 3 min at -20°C. Cells were incubated with pAb421 or pAb240 at 1 μg/ml following incubation in blocking solution [1% bovine serum albumin (BSA)/PBS] for 30 min. Coverslips were washed for 30 min with PBS and incubated for 45 min in fluorescein-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:500 in blocking solution. Coverslips were then washed for 30 min in PBS, mounted and viewed using a confocal microscope equipped with an Argon/Krypton laser (model LSM 410; Carl Zeiss, Inc., Thornwood, NY). Negative controls were incubated with 1 μg/ml normal mouse IgG substituted for pAb421 and pAb240.

PCR-SSCP analysis of p53 transcripts. Total cellular RNA (2 μg/15 μl) from MCF10A and MCF10AneoT-derived cells was reverse transcribed using oligo $(dT)_{15}$ primer. An aliquot of the reaction mixture was subjected directly to PCR using primer a^+, 5'-GTCACTGCCATGGAGGAGCCG-3' (base 113-133) (29), and primer t^-, 5'-TTATGGCGGGAGGTAG ACTG-3' (base 1244-1263) (29) to amplify *p53* cDNA. As a control, the same primers were used to amplify wild-type *p53* cDNA from the plasmid, pC53-SN3 (30). PCR was performed in 50 μl reaction mixtures containing 1 μl of RT-PCR template, 1 μM of each primer, 10 nM of each dNTP, 5 μCi of [α-^{32}P]-dCTP and 1X PCR buffer (Promega, Madison, WI). PCR was carried out for 35 cycles of 2 min at 95°C, 2 min at 59°C and 3 min at 68°C. A 5 μl sample of each reaction was subjected to electrophoresis on 1% agarose gel and radiolabeled products visualized by autoradiography. Individual portions of the ^{32}P-labeled PCR products were digested with restriction enzymes, AvaII, BsmAI, BsmI, MaeIII or StuI and subjected to electrophoresis on 2.5% agarose gels.

For single strand conformation polymorphism (SSCP) analysis, the restricted PCR fragments were diluted 9-fold in denaturation solution (95% $HCONH_2$, 20 mM EDTA, pH 8.0) and heated at 95°C for 5 min. The denatured samples were electrophoresed through 6% polyacrylamide gels in 0.5X Tris-borate buffer containing 1 mM EDTA at 4°C and 25 W constant power. Following electrophoresis, gels were dried and subjected to autoradiography.

Genomic DNAs isolated from MCF10A-derived cells and normal human placenta were subjected to hot start PCR using primers e^+ (5'-GATGCTGTCCCCGGACGATATT-3', base 250-271) (29) and f^- (5'-TTGGCTGTCCCAGAATGCAAG AA-3', base 458-480) (29). Radiolabeled PCR products were analyzed by SSCP as described above.

Analysis of DNA binding activity of P53

Preparation of cell extracts. MCF10A and MCF10AT system cells (MCF10AT1, MCF10AT2b, MCF10AT3b and MCF10AT3c) were plated on 100-mm dishes and grown to 75% confluency at 37°C. Whole cell extracts were prepared by three cycles of freezing and thawing in 0.1 ml of buffer containing 20 mM HEPES (pH 7.9), 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 0.4 M KCl, 1 mM PMSF, 1 μg/ml each of leupeptin, aprotinin, and pepstatin, and 20% glycerol. Extracts were dialyzed and clarified by centrifugation at 14,000 x g for 10 min and stored at -70°C. Protein concentrations were determined by the Bradford method (31).

DNA binding assay. Oligonucleotides containing the WAF1 P53 binding site (top strand, 5'- aagtggatccGAACATGTCCC AACATGTTg-3'; bottom strand, 5'-aggaagatctcAACATGTT GG-GACATGTTC-3) (17) or the vitamin D responsive element of the human osteocalcin gene (top strand, 5'-aagtgg atccTTGGTGACTCACCGGGTGAACGGGGGCATG-3'; bottom strand, 5'-aggaagatctCAATGCCCCCGTTCACC CGGTGAGTC-3') (32) were annealed and 5' overhangs were filled in with the Klenow fragment of *E.coli* DNA polymerase I and dATP, dGTP, dTTP and either dCTP (competitors) or [α-^{32}P]dCTP. Whole cell extracts were preincubated with 2 μg poly(dI-dC) and 0.5 μg mAb (pAb421 or pAb1801) for 30 min at room temperature in 20 μl of binding buffer (20 mM HEPES, pH 7.9, 1 mM DTT, 50 mM KCl, 5 mM $MgCl_2$, 10 μM $ZnSO_4$, 0.1 mg/ml BSA, 0.1% NP40, and 5% glycerol). 5 ng of [α-^{32}P]-labeled ds WAF1 P53 binding site was added and incubated for another 30 min at room temperature. Competitor oligonucleotides (either WAF1 P53 binding site or osteocalcin vitamin D response element) were added during the preincubation step. Reaction mixtures were analyzed by electrophoresis at 160 V on 6% polyacrylamide gels (80:1, acrylamide:bisacrylamide) in 0.5 X Tris-Borate buffer containing 1 mM EDTA under cooling by water circulation

(8-10°C). Following electrophoresis, gels were dried and autoradiographed.

Analysis of transcription activation function of P53

Plasmid constructs. The ß-galactosidase reporter plasmid, pWAF1/ßgal, was constructed by cloning a 2.4 HindIII WAF1 promoter fragment from pWAF1-Luc into Blue Script/ßgal (17). The wild-type *p53* expression vector pC53-SN3 (30) has a CMV immediate early promoter upstream of the coding sequences for wild-type *p53*. The plasmid, pCMV-neo is identical to pC53-SN3 except that it lacks the *p53* coding sequences.

Transfection. MCF10A, MCF10AneoT, MCF10AT1, and MCF10AT3b cells ($5x10^5$ cells per 60 mm dish) were plated 18 h prior to transfection in media as described before. To determine the functionality of endogenous P53, cells were transfected with varying amounts of pWAF1/ßgal (2.5-10 µg) using the calcium phosphate procedure (33). The effect of exogenous wild-type P53 on WAF1-induced reporter activity was determined by cotransfecting cells with 5 µg of WAF1/ßgal and varying amounts of pC53-SN3 (0.5-5 µg). Five µg of WAF1/ßgal was chosen since cells transfected with this amount of the plasmid yielded near maximal reporter activity that was still in the linear range. In both sets of transfection experiments, the control pCMVneo vector was used as carrier DNA to adjust the final concentration of DNA to 10 µg/ml. Cells were incubated with plasmid DNA for 4 h, followed by 3 min incubation with 20% glycerol. Cells were lysed 28 h following transfection and ß-gal activity assayed by ELISA (Boehringer Mannheim, Indianapolis, IN). The reporter plasmid pSVCAT (Promega, Madison, WI) was cotransfected with pWAF1/ßgal into MCF10A and MCF10AT derivatives in some experiments to monitor transfection efficiency. The efficiency of wild-type P53 expression from PC53-SN3 in the transfected cells was assessed by Western blot analysis.

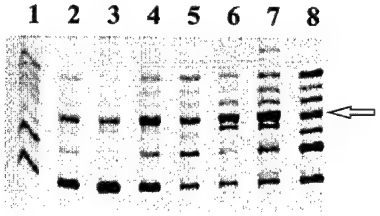

Figure 1. Synthesis of P53 in MCF10A and MCF10AT system cells. Cells were labeled with [^{35}S]-methionine for 3 h and processed for immunoprecipitation with P53-specific mAb pAb421 or normal mouse IgG. Precipitated proteins were analyzed by SDS-PAGE and visualized by fluorography. Note the appearance of enhanced labeling in the lower band of the P53 doublet in MCF10AT cells. Lane 1, MCF10AT2b immunoprecipitated with normal mouse IgG. Lanes 2-8 were immunoprecipitated with mAb pAb421. Lane 2, MCF10Aneo; lane 3, MCF10AneoN; lane 4, MCF10AneoT; lane 5, MCF10AT1; lane 6, MCF10AT2; lane 7, MCF10AT3b and lane 8, MCF10A.

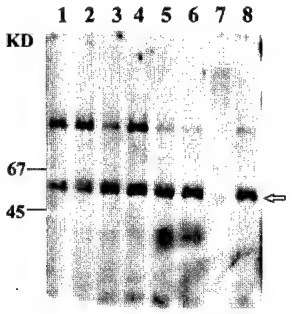

Figure 2. Synthesis and accumulation of conformationally altered P53 in MCF10AT system cells. [^{35}S]-methionine labeled proteins were immunoprecipitated with pAb240 or normal mouse IgG and precipitated proteins separated by SDS-PAGE. Lane 1, MCF10A; lane 2, MCF10AneoN; lane 3, MCF10AneoT; lane 4, MCF10AT1; lane 5, MCF10AT2b; lane 6, MCF10AT3b; lane 8, MCF10AT3c. Lane 7, labeled proteins of MCF10AT3b immunoprecipitated with normal mouse IgG.

Results and Discussion

P53 protein expression. The levels of P53 synthesized *de novo* were examined in exponentially growing cultures by metabolic labeling with [^{35}S]-methionine and immunoprecipitation with anti-P53 antibodies. Cells were incubated with [^{35}S]-methionine for 3 h to ensure achievement of equilibrium labeling in both rapidly degraded and stable forms of P53. MAbs used to detect P53 were pAb421 which binds to an epitope near the carboxyl-terminus: residues 371-380 of human P53 and is reactive to both wild-type and mutant forms, and pAb240 which recognizes a conserved amino acid motif RHSVV, residues 213-217 in human P53. This epitope is cryptic in wild-type P53 but exposed in many oncogenic mutants (34). Normal mouse IgG, used as a control, failed to precipitate 53 kDa proteins from MCF10AT cell lysates (Fig. 1, lane 1, a representative MCF10AT2b lysate is shown). With the exception of MCF10AT1 cells, the ratio of radiolabeled P53 immunoprecipitated with pAb421 relative to total radiolabeled lysate protein was significantly higher for MCF10AT system cells than for MCF10A, MCF10Aneo and MCF10AneoN cells (Fig. 1). Densitometric analysis of the radiolabeled P53 bands indicated 2-, 4- and 7-fold higher levels of P53 in MCF10AneoT, MCF10AT2b and MCF10AT3b cells, respectively, as compared to MCF10A, MCF10Aneo or MCF10AneoN cells. A significant portion of the increased radiolabel in pAb421 immunoprecipitable P53 was present in a form that migrated more rapidly than P53 from MCF10A, MCF10Aneo or MCF10AneoN cells. In order to determine whether the two forms of P53 represented wild-type and conformationally altered P53, the radiolabeled proteins in MCF10AT cells were immunoprecipitated with pAb240. This resulted in specific precipitation of a single species of P53 protein that was present in significantly higher amounts in MCF10AT cells than in MCF10A and MCF10AneoN cells (Fig. 2). This difference in ability to bind pAb421 and pAb240 indicates that the increase in levels of total P53 observed in MCF10AT system cells arises primarily from increased levels of conformationally altered wild-type P53. This could occur either by denaturation of wild-type P53 or by the presence of activating mutations (34,35).

Cellular distribution of P53. Immunofluorescence and confocal microscopy were used to visualize the distribution of P53

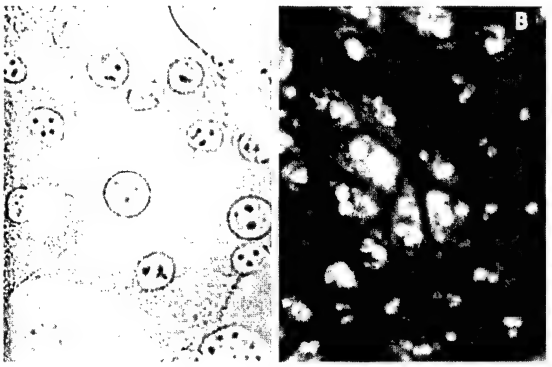

Figure 3. Distribution of P53 in nuclei and cytoplasm of confluent MCF10AT3b cells by immunofluorescence and confocal microscopy. Cells grown on coverslips were fixed in methanol-acetone (1:1, vol/vol) as described in Materials and methods. A, reactivity of P53 to pAb421 is restricted to nuclei of confluent MCF10AT3b cells that is visualized as punctate or stippled nuclei staining (indicated by arrows). B, reactivity of P53 to pAb240 is associated with staining of distinct large aggregates in both the nuclear and cytoplasmic compartments. Photomicrographs were made with a 40x objective.

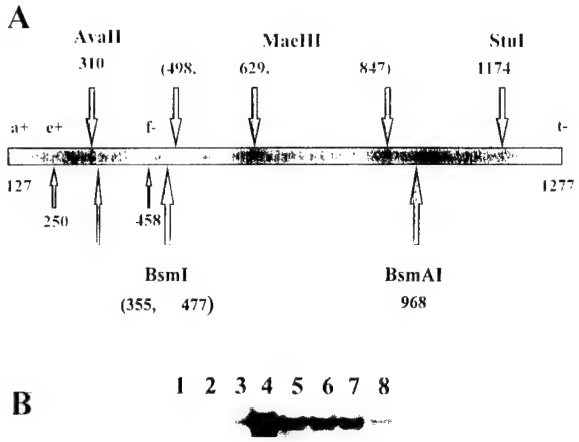

Figure 4. A, the position of primer pairs, a^+/t^-, and e^+/f^-, are indicated. Reverse transcribed cDNA products amplified by using primer pairs a^+ and t^- were digested with enzymes AvaII, BsmAI, BsmI, MaeIII or StuI as described in Materials and methods. B, SSCP analysis of *p53* gene and cDNAs amplified by the primer pair e^+/f^-. Lane 1, wild-type *p53* encoded plasmid pC53-SN3; lane 2, human placental genomic DNA; lanes 3-5, genomic DNA from MCF10A, MCF10AneoT and MCF10AT3b, respectively; lanes 6-8, *p53* cDNAs amplified from MCF10A, MCF10AneoT and MCF10AT3b, respectively.

(reactive to pAb421 and pAb240 antibodies) in the nuclei and cytoplasm of MCF10AT3b cells. MCF10AT3b cells were chosen since they represented the most advanced serial xenograft passage of MCF10AT system cells, and metabolic labeling experiments have shown these cells to express significant amounts of conformationally altered P53 (Figs. 1 and 2). Results of immunofluorescence microscopy confirm the abundant presence of pAb240-reactive P53 in >95% of MCF10AT3b cells. Staining is often associated with large distinct aggregates in both the nuclear and cytoplasmic compartments (Fig. 3B). Similar accumulation of pAb240 reactive P53 has been reported in MCF-7 cells, suggesting a correlation between increasing accumulation of conformationally altered P53 and increasing potential for tumorigenicity (36). In contrast to pAb240 reactivity, pAb421 reactive protein is exclusively present in the nuclei of confluent MCF10AT3b cells; i.e., >90% of nuclei show punctate or granular staining (Fig. 3A). While pAb421 reactive P53 includes both native and conformationally altered forms in exponentially growing MCF10AT cells (Fig. 1), this reactivity is clearly altered in confluent cells possibly due to modifications that influence pAb421 immunoreactivity.

SSCP analysis of P53 mRNAs amplified from MCF10AT system cells. Since both accumulation of P53 and alteration in conformation as recognized by pAb240 could result from point mutations in the coding region of *p53*, *p53* transcripts amplified from MCF10AT system cells were analyzed for sequence variation by the SSCP method. Total cellular RNA from MCF10A and MCF10AT system cells was transcribed into cDNA using AMV reverse transcriptase and oligo $(dT)_{15}$. The resultant single stranded cDNA fragments were amplified by PCR as radiolabeled double stranded DNA fragments using a pair of oligonucleotide primers and [α-^{32}P]-dCTP. PCR amplified fragments from MCF10A and MCF10AT system cells were compared to similarly amplified fragments from human wild-type *p53* cDNA encoded in the plasmid pC53-SN3 (30). Amplification of *p53* cDNA using primers a^+ and t^- yielded a fragment with a nucleotide length of 1151 bp which was identical to that obtained from the wild-type *p53* cDNA, indicating that no major deletions had occurred in the coding regions. In order to analyze for conformational polymorphism arising from point mutations, the amplified cDNA fragments were subjected to restriction digestion with MaeIII, BsmI, BsmAI, StuI or AvaII to obtain overlapping fragments of suitable size for SSCP analysis. The positions of the primers and restriction enzymes used for SSCP analysis are illustrated in Fig. 4A. Restriction fragments derived from and including all of the sequences in exons 1-3 and 5-11 had identical mobilities regardless of whether they were prepared from *p53* cDNAs of MCF10A cells, MCF10AT system cells or the wild-type *p53* cDNA encoded in the plasmid pC53-SN3 (data not shown). However, the mobility of restriction fragments spanning exon 4 that were derived from control wild-type *p53* cDNA differed from those of similar fragments derived from MCF10A and MCF10AT cDNAs. These results were verified by SSCP analysis of exon 4 sequences directly amplified by PCR using primers e^+ and f^- (base 250-480) (29). The mobilities of the exon 4 sequences from genomic DNAs of human placenta, MCF10A, MCF10AneoT or MCF10AT3b cells were identical to each other and to exon 4 sequences from MCF10A, MCF10AneoT or MCF10AT3b cDNAs but differed from those of exon 4 sequences of wild-type *p53* cDNA encoded in the plasmid pC53-SN3 (Fig. 4B). Thus, all MCF10A and MCF10AT system cells analyzed express only one allelic form of *p53*. Sequencing of exon 4 DNA confirmed that its sequence was indeed normal in the *p53* gene of MCF10A and MCF10AT system cells, and that the exon 4 alteration observed in pC53-SN3 p53 cDNA represented a previously characterized allelic polymorphism (CGC→CCC) at codon 72 (37).

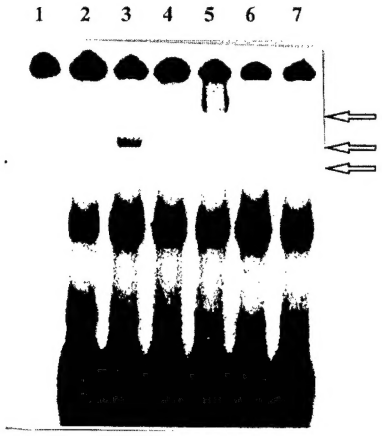

Figure 5. P53 from MCF10A cells exhibit sequence-specific DNA binding. Whole cell extracts were prepared from MCF10A cells as described in Materials and methods and assayed for sequence-specific DNA binding by electrophoretic mobility shift assay using the WAF1 P53 binding site as the oligonucleotide probe. MAbs pAb421 or pAb1801 were added as indicated, and the protein-DNA complexes were resolved on a native 6% polyacrylamide gel. Competitor oligonucleotides were added at molar excess; the vitamin D response element was the nonspecific competitor used. Lane 1, free WAF1 DNA probe; lane 2, binding in absence of antibodies; lane 3, binding in presence of pAb421; lane 4, binding in presence of pAb421 and 100X unlabeled WAF1 DNA; lane 5, binding in presence of pAb421 and pAb1801; lane 6, binding in presence of pAb1801 and lane 7, binding in presence of pAb1801 and 100X unlabeled nonspecific competitor.

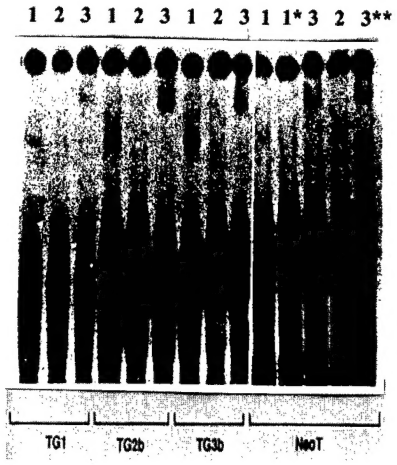

Figure 6. Sequence-specific DNA binding activity of P53 from MCF10AT xenograft derived cell lines is defective but can be restored with pAb421. Whole cell extracts were prepared from MCF10AT xenografts and assayed for sequence-specific DNA binding by electrophoretic mobility shift assay as described in Materials and methods. MAbs pAb421 or pAb1801 were added as indicated, and the protein-DNA complexes were resolved on a native 6% polyacrylamide gel. Radioinert WAF1-P53 binding site and vitamin D response element added in molar excess were included as specific and nonspecific competitors, respectively. Lane 1, binding in presence of pAb421; lane 2, binding in presence of pAb1801; lane 3, binding in presence of pAb421 and 1801. *Binding in presence of 100-fold excess unlabeled specific competitor and **binding in presence of 100-fold excess nonspecific competitor.

These results indicate that mutation in *p53* does not play a major role in progression in the MCF10AT model system. Instead, they suggest that changes in the balance between normal and altered conformation of wild-type P53 might provide a mechanistic basis for altered cell division.

To further examine this possibility we analyzed the relationship between levels of pAb240-reactive P53 and P53 capacity for site-specific DNA binding and transcriptional activation.

Analysis of DNA binding function of P53 expressed in MCF10AT system cells. The ability of wild-type P53 to cause G1 arrest *in vivo* is believed to require sequence-specific binding to DNA. Wild-type P53, but not mutant P53 (alterations in the DNA binding domain), binds *in vitro* to sequences matching the consensus 5'-$(Pu)_3$ C(A/T)(A/T) $G(Py)_3$-3' (8). To detect the presence of proteins with capacity to bind the P53 recognition site in the promoter of the WAF-1 gene (17), whole cell extracts of proteins from MCF10A, MCF10AneoT, MCF10AT1, MCF10AT2b, MCF10AT3b and MCF10AT3c cells were tested for binding to a double stranded oligonucleotide containing this site by electrophoretic mobility shift assay. Proteins in whole cell extracts from MCF10A cells gave a low but detectable level of complex formation with the P53 binding site (Fig. 5, lane 2). Addition of either pAb421 or pAb1801 to binding reactions enhanced formation of protein:DNA complexes, although pAb421 was much more efficient in forming a 'supershifted' complex (compare Fig. 5, lane 3 and lane 6). This suggested that both antibodies are binding to P53 complexed with the P53 recognition site in DNA, but that pAb421 is much more efficient in stabilizing the P53:DNA complex. This was confirmed by observation of an additional decrease in the mobility (but not the amount) of the protein:DNA complex when both pAb1801 and pAb421 were present in the binding reaction (Fig. 5, lane 5). Specificity was confirmed by determining that unlabeled oligonucleotides containing the P53 binding site competed effectively with radiolabeled P53 in the formation of the pAb421:P53:DNA complex (Fig. 5, lane 4) while a nonspecific oligonucleotide competitor containing the vitamin D response element had no effect on complex formation (Fig. 5, lane 7). Protein in extracts from MCF10AneoT cells was similar to protein from MCF10A cells in its capacity to form P53:DNA complexes (data not shown). However, proteins in extracts from MCF10AT xenograft derived cell lines did not form detectable complexes with the WAF-1 P53 binding site (data not shown) unless pAb421 was also present in the binding mixture (Fig. 6, lane 1). pAb1801 had no stabilizing effect on P53 binding (Fig. 6, lane 2). These results indicate that in both MCF10A and MCF10AneoT cells, a portion of native P53 is dependent on pAb421 for complex formation with the P53 binding site and that this portion is increased in cell lines derived from MCF10AT xenografts where all P53:DNA complex formation requires the presence of pAb421. Since the ratio of conformationally altered P53:normal P53 is much higher in cells of MCF10AT xenograft derived lines than in MCF10A and MCF10AneoT cells, these results imply an inverse relationship between availability of native P53 for DNA binding and level of conformationally altered wild-type P53.

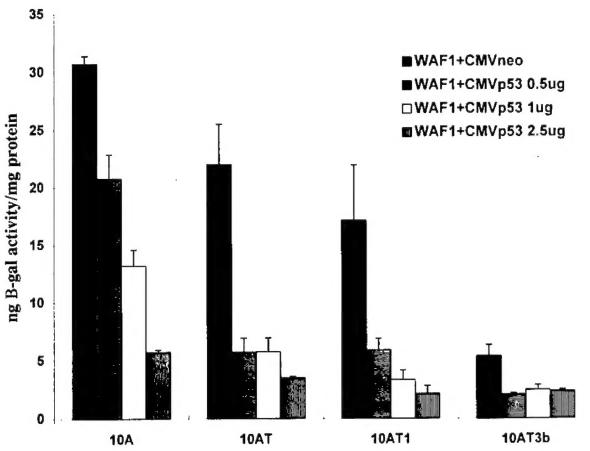

Figure 7. Functionality of P53 by reporter gene expression from a transiently transfected WAF1/ßgal construct in MCF10A and MCF10AT system cells. Cells were transfected with pWAF1/ßgal alone (5 µg) or in combination with pC53-SN3 (0.5-2.5 µg), an expression vector for wild-type P53 under the control of CMV promoter. The total amount of DNA in all samples were maintained at 10 µg with pCMV-neo as carrier DNA. Cell lysates prepared 28 h after transfection were analyzed for ß-gal (reporter) activity by ELISA. Results obtained from three independent experiments are summarized and graphically represented. Values are expressed as mean ± SD. Cotransfection of cells with pSVCAT and pWAF1/ßgal yielded comparable levels of CAT activity confirming that the results were not due to differences in transfection efficiency or cell viability (data not shown).

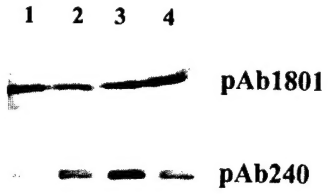

Figure 8. Immunological reactivity to mAbs pAb1801 and pAb240 of P53 from cell lysates of pC53-SN3 and pWAF1/ßgal co-transfected MCF10A and MCF10AT system cells. 28 h following transfection, cell lysates equivalent to 50 µg protein were immunoprecipitated with pAb1801 or pAb240 and precipitated proteins subjected to Western blot analysis with a polyclonal sheep anti-P53 antibody, Ab-7. Lane 1, MCF10A; lane 2, MCF10AneoT; lane 3, MCF10AT1 and lane 4, MCF10AT3b.

In this regard, it is of interest to note that pAb421 has been reported to activate the DNA binding function of P53 by mediating an allosteric change in its carboxyl-terminal regulatory domain (38).

Evaluation of functional status of P53 in the MCF10AT system. To determine whether the differences in capacity for formation of P53 complexes *in vitro* with the P53 recognition sequence in the WAF-1 promoter were related to P53 function as a transcription factor *in vivo*, MCF10A, MCF10AneoT, MCF10AT1, and MCF10AT3b cells were transfected with the WAF-1 ß-galactosidase expression vector, pWAF1/ß-gal. As shown in Fig. 7, a significant decrease in the ability of endogenous P53 to transactivate ß-gal expression from the WAF-1 promoter was observed in all MCF10AT system cells as compared to MCF10A cells. The capacity for ß-gal expression appeared to decrease relative to the number of transplant generations of the xenografts from which the cells were derived (compare MCF10AT1 and MCF10AT3b data in Fig. 7). The observed differences in ß-gal expression are not related to variations in transfection efficiency or cell viability as MCF10A and MCF10AT3b cells cotransfected with pWAF1/ßgal and pSVCAT yielded approximately similar CAT activities (data not shown). Thus, although the MCF10AT system cells express higher levels of total P53 than MCF10A cells, the ability of this P53 to function as a transcription factor is significantly reduced, presumably because of its existence in a conformationally altered state with reduced DNA binding capacity.

To determine whether increasing the levels of wild-type P53 in MCF10AT system cells could 'rescue' their capacity for activating reporter gene expression from the WAF1 promoter, MCF10A and MCF10AT system cells were cotransfected with pWAF1/ßgal and various amounts of the wild-type P53 expression vector, pC53-SN3 (30). The results presented in Fig. 7 demonstrate that introduction of exogenous wild-type P53 did not enhance reporter activity over that mediated by endogenous P53 in any of the cell lines. In fact, measurable ß-gal activity was reduced in all cells in a dose dependent manner. Nevertheless, the data show the same inverse relationship between level of P53 mediated reporter gene activation and level of conformationally altered P53 relative to native P53, i.e., comparison of cells co-transfected with pC53-SN3 and pWAF1/ßgal showed that MCF10A cells had the highest level of P53-mediated reporter activity and MCF10AT3b cells the lowest (Fig. 7). They also indicate that levels of wild-type P53 are not limiting since introduction of exogenous wild-type P53 fails to enhance ß-gal expression from the WAF1 promoter.

It should be noted that inhibition of reporter gene expression occurred independently of any transcription factor depletion by the transfected CMV promoter, since all cells received an equivalent amount of this promoter in the form of pC53-SN3 or pCMVneo. Further, it was not due to loss of cell viability, which was only observed with levels of pC53-SN3 >5 µg. The observed differences in reporter gene activity were not related to variations in transfection or expression efficiency of pC53-SN3. Western blot analysis of cell lysates (50 µg protein) prepared from transfected cells revealed similar levels of pAb1801-reactive P53 (Fig. 8).

One possible explanation for the suppressive effect of increased wild-type P53 expression in MCF10 cells is induction of endogenous WAF-1 which down-regulates its own expression and could also down-regulate the response of the WAF-1 reporter plasmid. Alternatively, overexpression of P53 could predispose to increased ratios of conformationally altered:native P53. To examine this possibility, proteins were immunoprecipitated from cell lysates of pC53-SN3 co-transfected MCF10A and MCF10AT system cells with pAb240. Lysates from MCF10AT system cells still contained a higher concentration of P53 recognized by pAb240 than did lysates from MCF10A cells (Fig. 8). Thus, despite increased expression of exogenous wild-type P53, the pAb240-reactive conformationally altered P53 remains the predominant form in MCF10AT xenograft derived cell lines and may even represent

a mixture of endogenous and exogenous P53 that has undergone the same conformational alteration. This would suggest that conformationally altered wild-type P53 is not only functionally defective but may also able to interfere with the function of native wild-type P53 by sequestering it in heteromeric complexes. An alternate possibility is that other proteins or factors in the cellular milieu of MCF10AT system cells are conducive for inducing exogenous P53 to adopt a conformationally altered state that has a 'mutant' immunological phenotype.

Results in literature are not consistent regarding the prognostic role and pathogenetic involvement of P53 in early human breast cancer. While all of the studies reported thus far have examined structure and function of P53 synthesized *in vitro*, our findings with cell lines from the MCF10AT xenograft model for early breast cancer substantiate the existence of mechanisms that modulate the DNA binding function of P53 through effects on protein conformation that are independent of genetic alterations of *p53*. Although previous studies have described P53 proteins capable of reversibly assuming wild-type and 'mutant' conformations (39-41), our study is the first to provide evidence that conformationally altered wild-type P53 is functionally defective in transcriptional activation *in vivo*, a property that is essential for growth suppressive function of P53. Our results suggest that in MCF10AT xenografts and probably in a subset of breast tumors that show overexpression of wild-type P53, factors exist that favor the abnormal stabilization of wild-type P53 in a conformationally altered state which is defective in P53-mediated functions and interferes with regulation of proliferation, apoptosis and response to chemotherapy (42). While the nature of molecular forces that render P53 inactive in the MCF10AT xenograft model remains to be established, several mechanisms have been suggested that may potentially modulate P53 structure and function. In some tumors, latency of P53 has been shown to be maintained through retention of P53 in the cytoplasm, and activation results in transportation to the nucleus. The heat shock protein Hsp84 (43) and the C-terminal domain of P53 (44) have been suggested to play a role in cytoplasmic sequestration and nuclear transportation. Both of these mechanisms may be contributing to inactivation of P53 in exponentially growing MCF10AT derived cell lines, since our immunofluorescence studies indicate abnormal accumulation of conformationally altered P53 in nuclei and cytoplasm.

Other mechanisms that have been suggested to render non-mutated wild-type P53 inactive *in vivo* include overexpression of zinc chelators such as metallothionein (MT) (45). In this regard, it is of interest to note that overexpression of MT is frequently observed in breast cancers carrying wild-type *p53* alleles (46,47). Another important mechanism for P53 regulation has been reported to result from redox regulation and metal binding. Redox modulators that reversibly perturb the wild-type phenotype of P53 can influence the biological activity of P53 by modulating DNA binding through effects on protein conformation (48). Similarly, P53 protein conformation and activity can be modulated through phosphorylation. Although phosphorylation at the C-terminus is not a prerequisite for conferring DNA binding capacity, it represents the most physiologically relevant mechanism by which the conformation of P53 may be altered to confer DNA binding activity. In fact, mutation of a C-terminal serine residue of murine P53 has been shown to eliminate its tumor suppressive activity in mammalian cells, suggesting that phosphorylation of this residue is required for activation of P53 tumor suppression function (49). The accumulation of pAb240-reactive P53 in cytoplasm and nuclear compartments of MCF10AT cells, suggest that wild-type P53 inactivation may be occurring via one or more of these mechanisms.

In summary, our results clearly indicate that *p53* mutations are not necessary to account for *p53*-mediated changes in growth and morphology that occur in the early preneoplastic stages of breast tumor progression in the MCF10AT xenograft model. Rather, they indicate that stable changes in wild-type P53 conformation and function play a major role in breast cancer development. The availability of the MCF10AT xenograft model should provide an opportunity to further elucidate some mechanisms by which P53 function is altered and the consequences of these alterations in early stages of breast cancer which are technically difficult to examine in human tissues.

Acknowledgments

We thank Dr P. Abarzua for his gift of pWAF1/ßgal and Dr B. Vogelstein for pC53-SN3. We also thank Drs Avraham Raz and Sandra Wolman for critical reading of the manuscript and helpful suggestions. The research was supported by grants from the U.S. Army Medical Research and Materiel Command (DAMD17-94-J-4427) and National Institutes of Health (CA60881 and CA22453). The Confocal Imaging Core Facility studies were supported in part by Center grants P30ES06639 from National Institute of Environmental Health Sciences and P30CA22453 from the National Cancer Institute. Robert Welte was supported by a Freiburg Exchange Scholarship.

References

1. Momand J, Zambetti GP, Olson DC, George DL and Levine AJ: The mdm2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 69: 1237-1245, 1992.
2. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM and Bos JL: Genetic alterations during colorectal tumor development. N Engl J Med 319: 525-532, 1988.
3. Bennett WP, Hollstein MC, Metcalf RA, Welsh JA, Zhu SM, Kusters I, Resau JH, Trump BF, Lane DP and Harris CC: P53 mutation and protein accumulation during multistage human esophageal carcinogenesis. Cancer Res 52: 6092-6097, 1992.
4. Sugimoto K, Toyoshima H, Sakai R, Miyagawa K, Hagiwara K, Hirai H, Ishikawa F and Takaku F: Mutations of the p53 gene in lymphoid leukemia. Blood 77: 1153-1156, 1991.
5. Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T, Hovig E, Smith-Sorensen B, Montesano R and Harris CC: Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res 22 (Suppl.): 3551-3555, 1994.
6. Fields S and Jang SK: Presence of a potent transcription activating sequence in the p53 protein. Science 249: 1046-1049, 1990.
7. Pietenpol JA, Tokino T, Thiagalingam S, El-Deiry WS, Kinzler KW and Vogelstein B: Sequence-specific transcriptional activation is essential for growth suppression by p53. Proc Natl Acad Sci USA 91: 1998-2002, 1994.
8. Kern SE, Kinzler KW, Bruskin A, Jarosz D, Friedman P, Prives C and Vogelstein B: Identification of p53 as a sequence-specific DNA-binding protein. Science 252: 1708-1711, 1991.

9. El-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW and Vogelstein B: Definition of a consensus binding site for p53. Nat Genet 1: 45-49, 1992.
10. Funk WD, Pak DT, Karas RH, Wright WE and Shay JW: A transcriptionally active DNA-binding site for human p53 protein complexes. Mol Cell Biol 12: 2866-2871, 1992.
11. Zambetti GP, Bargonetti J, Walker K, Prives C and Levine AJ: Wild-type p53 mediates positive regulation of gene expression through a specific DNA sequence element. Gene Dev 6: 1143-1152, 1992.
12. Farmer G, Bargonetti J, Zhu H, Friedman P, Prywes R and Prives C: Wild-type p53 activates transcription *in vitro*. Nature 358: 83-86, 1992.
13. Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW and Vogelstein B: Oncogenic forms of p53 inhibit p53-regulated gene expression. Science 256: 827-830, 1992.
14. Weintraub H, Hauschka S and Tapscott SJ: The MCK enhancer contains a p53 responsive element. Proc Natl Acad Sci USA 88: 4570-4571, 1991.
15. Barak Y, Juven T, Haffner R and Oren M: mdm2 expression is induced by wild-type p53 activity. EMBO J 12: 461-468, 1993.
16. Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace AJ Jr: A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71: 587-597, 1992.
17. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817-825, 1993.
18. Deb SP, Munoz RM, Brown DR, Subler MA and Deb S: Wild-type human p53 activates the human epidermal growth factor receptor promoter. Oncogene 9: 1341-1349, 1994.
19. Okamoto K and Beach D: Cyclin G is a transcriptional target of the p53 tumor suppressor protein. EMBO J 13: 4816-4822. 1994.
20. Martinez J, Georgoff I, Martinez J and Levine AJ: Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Gene Dev 5: 151-159, 1991.
21. Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A and Oren M: Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature 352: 345-347, 1991.
22. Lowe SW, Schmitt EM, Smith SW, Osborne BA and Jacks T: p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362: 847-849, 1993.
23. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML and Wyllie AH: Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 362: 849-852, 1993.
24. Basolo F, Elliott J, Tait L, Chen-XQ, Maloney T, Russo IH, Pauley R, Momiki S, Caamano J, Klein-Szanto AJ and Russo J: Transformation of human breast epithelial cells by c-*Ha-ras* oncogene. Mol Carcinogen 4: 25-35, 1991.
25. Miller FR, Soule HD, Tait L, Pauley RJ, Wolman SR, Dawson PJ and Heppner GH: Xenograft model of progressive human proliferative breast disease. J Natl Cancer Inst 85: 1725-1732, 1993.
26. Dawson PJ, Wolman SR, Tait L, Heppner GH and Miller FR: MCF10AT: a model for the evolution of cancer from proliferative breast disease. Am J Pathol 148: 313-319, 1996.
27. Harlow E, Crawford LV, Pim DC and Williamson NM: Monoclonal antibodies specific for simian virus 40 tumor antigens. J Virol 39: 861-869, 1981.
28. Gannon JV, Greaves R, Iggo R and Lane DP: Activating mutations in p53 produce a common conformational effect: a monoclonal antibody specific for the mutant form. EMBO J 9: 1595-1602, 1990.
29. Locus HUMTP53B, Accession M14695.
30. Baker SJ, Markowitz S, Fearon ER, Willson JK and Vogelstein B: Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 249: 912-915, 1990.
31. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976.
32. Uchida M, Ozono K and Pike JW: Activation of the human osteocalcin gene by 24R, 25-di(OH)vitamin D3 occurs through the vitamin D receptor and the vitamin D responsive element. J Bone Miner Res 9: 1981-1987, 1994.
33. Graham FL and van der Eb AJ: A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52: 456-467, 1973.
34. Stephen CW and Lane DP: Mutant conformation of p53: precise epitope mapping using a filamentous phage epitope library. J Mol Biol 225: 577-583, 1992.
35. Finlay CA, Hinds PW, Tan TH, Eliyahu D, Oren M and Levine AJ: Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol Cell Biol 8: 531-539, 1988.
36. Bartek J, Iggo R, Gannon J and Lane DP: Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene 5: 893-899, 1990.
37. Matlashewski GJ, Tuck S, Pim D, Lamb P, Schneider J and Crawford LV: Primary structure polymorphism at amino acid 72 of human p53. Mol Cell Biol 7: 961-963, 1987.
38. Hupp TR, Meek DW, Midgley CA and Lane DP: Regulation of the specific DNA binding function of p53. Cell 71: 875-886, 1992.
39. Zhu YM, Bradbury DA and Russell NH: Wild-type p53 is required for apoptosis induced by growth factor deprivation in factor-dependent leukaemic cells. Br J Cancer 69: 468-472, 1994.
40. Milner J: A conformation hypothesis for the suppressor and promoter functions of p53 in cell growth control and in cancer. Proc R Soc Lond (Biol) 245: 139-145, 1991.
41. Hainaut P, Butcher S and Milner J: Temperature sensitivity for conformation is an intrinsic property of wild-type p53. Br J Cancer 71: 227-231, 1995.
42. Levine AJ: P53, the cellular gatekeeper for growth and division. Cell 88: 323-331, 1997.
43. Sepehrnia B, Paz IB, Dasgupta G and Momand J: Heat shock protein 84 forms a complex with mutant p53 protein predominantly within a cytoplasmic compartment of the cell. J Biol Chem 271: 15084-15090, 1996.
44. Ostermeyer AG, Runko E, Winkfield B, Ahn B and Moll UM: Cytoplasmically sequestered wild-type p53 protein in neuroblastoma is relocated to the nucleus by a C-terminal peptide. Proc Natl Acad Sci USA 93: 15190-15194, 1996.
45. Hainaut P and Milner J: A structural role for metal ions in the 'wild-type' conformation of the tumor suppressor protein p53. Cancer Res 53: 1739-1742, 1993.
46. Oyama T, Take H, Hikino T, Iino Y and Nakajima T: Immunohistochemical expression of metallothionein in invasive breast cancer in relation to proliferative activity, histology and prognosis. Oncology 53: 112-117, 1996.
47. Goulding H, Jasani B, Pereira H, Reid A, Galea M, Bell JA, Elston CW, Robertson JF, Blamey RW, Nicholson RA, Schmid KW and Ellis IO: Metallothionein expression in human breast cancer. Br J Cancer 72: 968-972, 1995.
48. Hainaut P and Milner J: Redox modulation of p53 conformation and sequence-specific DNA binding *in vitro*. Cancer Res 53: 4469-4473, 1993.
49. Milne DM, Palmer RH and Meek DW: Mutation of the casein kinase II phosphorylation site abolishes the anti-proliferative activity of p53. Nucleic Acids Res 20: 5565-5570, 1992.